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A MICROSCOPIC BIOASSAY FOR MELATONIN[1]

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ABSTRACT

A microscopic bioassay procedure for the determination of melatonin is described, and factors influencing the test are discussed. The techniques employed can be used to assay for other agents which produce lightening of frog skin as well as for compounds that cause darkening.

MANY substances, including various hormones, can bring about a change in the color of frog skin (1). Darkening results from dispersion of pigment granules throughout the cytoplasm of melanocytes, and lightening occurs when the pigment granules become aggregated about the nuclei of the cells. Several procedures have been devised to quantitate the darkening and lightening reactions. Among microscopic methods the assay developed by Hogben and Slome (2) to obtain a melanocyte index is well known. A photometric method measuring light transmitted through frog skin was described by Wright (3) and by Frieden, Fishbein and Hisaw (4). Subsequently, an improved photometric method utilizing measurement of light reflected from frog skin was developed by Shizume, Lerner and Fitzpatrick (5). Recently this type of bioassay was improved further (6).

The pineal gland was found to contain a very potent lightening hormone, melatonin, which is N-acetyl-5-methoxytryptamine (7, 8). In the isolation of melatonin it was desirable to combine microscopic and photometric techniques in a bioassay procedure that was quantitative, rather specific and easy to do. The bioassay procedure used in the isolation work is reported here.

BIOASSAY PROCEDURE

Frogs (*Rana pipiens*) were killed by pithing, and the skin was stripped from the median-dorsal surface of the thigh and placed in Ringer's solution. One piece of skin was

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obtained from each leg. The Ringer's solution was prepared by dissolving 6.5 gm. NaCl, 140 mg. KCl, 120 mg. $CaCl_2$ and 200 mg. $NaHCO_3$ in distilled water which was made up to a final volume of 1 liter. The skin was stretched over the center ring in a small dish, and an outer ring like an embroidery hoop was placed over the skin to fix it in place. Thirty ml. of Ringer's solution was added to each dish to immerse the skin.

The dishes were made of transparent plastic as shown in Figure 1. A piece of plastic with a hole large enough to support a ring rigidly was glued to the bottom of the dish. The ring was approximately one inch in diameter and $\frac{3}{8}$ of an inch high. Holes were drilled in the ring to allow for free circulation of the surrounding media. The sets were made so

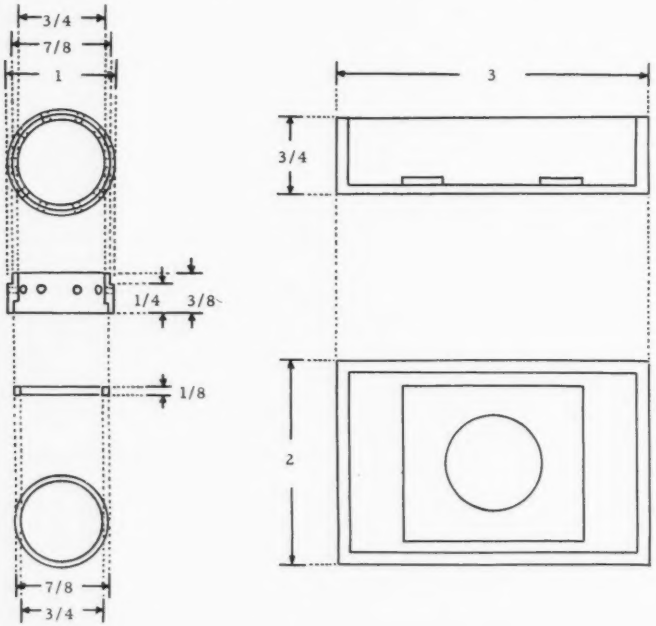

FIG. 1. Dishes and rings for the assay were made of transparent plastic. Dimensions are given in inches.

that all parts could be interchanged. A syringe with a curved needle that could be inserted into the holes of the center ring was useful for removing air bubbles captured under the extended skin.

To measure photometrically the opacity of melanocytes of frog skin stretched over the ring, a microscope with an attached photocell was used (Fig. 2). Our instrument was a Leitz Ortholux microscope with a calibrated stage that permitted the use of light transmitted through or reflected from cells. However, a simpler arrangement also would be satisfactory. The intensity of light from the pigment cells was indicated on a photometer. Generally, transmitted and not reflected light was used. For most of the assays it was simplest to work with a 10× objective lens and observe about 50 melanocytes at one time. However, it was possible to employ a 75× objective lens and observe changes in only a single cell. Our standard light source was light passed through a dark green glass filter. The filter was placed on the mechanical stage and the intensity of light emerging

om the filter was checked on the photometer. A voltage stablizer was helpful in obtaining a light source of constant intensity. For determination of a substance like melatonin that lightens pigment cells it was necessary to begin the assay with dark cells and then measure the degree of lightening induced by melatonin. The skin could be darkened with caffeine or MSH. Usually 3.0 ml. of 1% caffeine in Ringer's solution was added to each dish. After 90 minutes maximal darkening had occurred, and the skin was ready for bioassay. The darkened skin was placed on the mechanical stage, and five fields were selected from each skin specimen. The location of each field was recorded accurately on the two scales of the calibrated stage. The fields selected were required to satisfy the

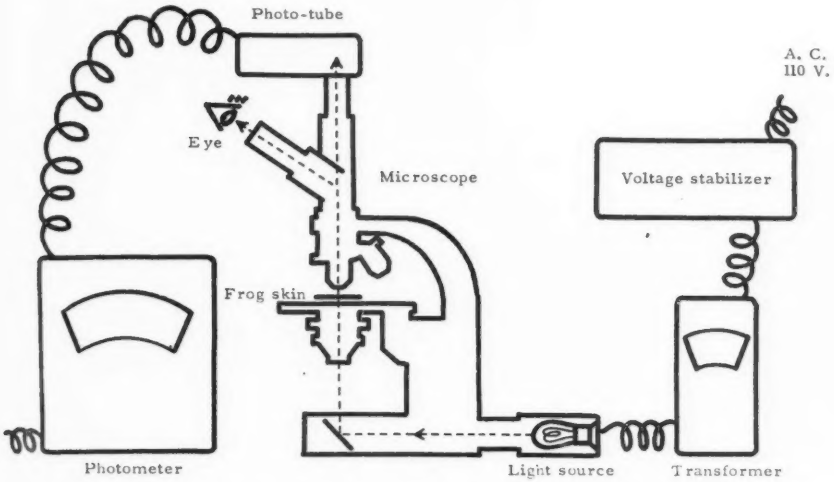

FIG. 2. A Leitz Ortholux microscope, a Photovolt Model 520 M photometer and a voltage stabilizer as shown schematically were used.

following conditions:

1. The population density of melanocytes must be relatively large, about 50 cells per field.
2. The melanocytes should be in the fully dispersed phase.
3. Few cells other than melanocytes can be present.
4. The skin must be relatively thin.

A photometric reading was made of each field in all the dishes. Solutions containing unknown amounts of melatonin were made up in serial dilutions of 1 to 10 or 1 to 3. The minimum effective dose that would produce an increase in light transmission was sought. Initially solutions were made up in dilutions of 1:10, 1:100, 1:1000 and 1:10,000. When the minimum effective concentration was found, smaller dilution steps were made, e.g., 1:3 1:9, 1:27 and 1:81; and again the minimum effective concentration was found. When necessary other dilution ratios were used.

The quantity of melatonin per milliliter that produces a minimum increase in light transmission represents a unit of melatonin. If frog skin is in 30 ml. of Ringer's solution, 30 units of melatonin are required for the minimum effect. The number of units in a sample is determined from the dilution necessary to produce minimum lightening. That

is, if a solution must be diluted 100,000 times to give the required response, the sample is said to contain 100,000 units of melatonin.

The actual assay can be carried out in several ways. When accurate values of melatonin levels are needed, it is best to use the skin from one thigh of a frog for the unknown sample and skin from the other thigh for the standard solution. Skin specimens obtained from the right and left legs of the same frog give a more similar response than samples from different frogs. However, when only approximate values of melatonin levels are needed, it is not necessary to run a standard sample with the unknown. If nothing is known about the melatonin concentration of a sample, unmatched skins are used.

The assay was begun by adding 0.3 ml. of the most dilute solution to a dish containing darkend skin and 30 ml. of Ringer's solution. After adequate stirring the solution was allowed to stand for 30 minutes. Readings were then taken again on the original five fields. If a significant change occurred, it was necessary to dilute the solutions more. If there was no change, or if the change was very small, the next most concentrated solution was added. However, before adding the more concentrated solution, it was necessary to subtract the quantity of melatonin previously added from the 0.3 ml. to be added. Thus the amount added was

$$0.3 - \frac{0.3}{n}$$

where n is the dilution factor which is usually 10 or 3. That is, when the serial dilutions 1:10, 1:100, 1:1000, etc. are used, $n=10$; and when the dilutions are 1:3, 1:9, 1:27, etc., $n=3$. When n was larger than 10 this correction factor was omitted because it was negligible.

After finding the appropriate concentration for lightening, or after the most concentrated solution was added and no change occurred, the skin was tested for its lightening capacity by adding 1 ml. of 0.01% l-noradrenaline bitartrate in Ringer's solution to each dish. This quantity of noradrenaline was sufficient to produce maximum aggregation of pigment granules. An excess of melatonin may be added instead of noradrenaline. After 30 minutes, photometer readings weret aken again. The ratio of the difference in photometer readings for the unknown (R) and that for maximum dispersion (darkening) M_d to the difference in readings for maximum aggregation (lightening) M_a and maximum dispersion M_d was determined.

$$\frac{R - M_d}{M_a - M_d} = \frac{\Delta_1}{\Delta_2}$$

Five values for the ratio Δ_1/Δ_2 were obtained from each skin sample. When the mean value for this ratio became 0.2, the minimum effective response had been obtained.

Usually the reading for maximum dispersion, M_d, was the photometer reading at the beginning of the assay after the skin had been darkened with caffeine. However, if the first few samples added to the dish had no melatonin, the skin might continue to darken a little. When this happened, the smallest reading was used for M_d. The reading for maximum aggregation, M_a, usually was the photometer value at the end of the experiment after noradrenaline had been added. However, sometimes a melatonin sample produced greater light transmission than did noradrenaline used at the end of the experiment. When this happened the largest value was used for M_a.

If none of the dilutions gave a value of 0.2 for Δ_1/Δ_2, the dilution value of the sample was plotted on the logarithm scale of semi-logarithm paper against Δ_1/Δ_2 and the dilution value necessary to make Δ_1/Δ_2 equal to 0.2 was obtained by extrapolation.

When the approximate number of melatonin units was found for a sample, a more accurate assay was carried out by comparing the effect of dilutions of the unknown on skin from a frog's leg with the effect of a standard quantity of melatonin on skin from the other leg of the same frog. Sometimes it was desirable to plot the log of the dilution

values against Δ_1/Δ_2 for both the unknown and the standard solution in order to obtain very accurate results.

Approximately 300,000 units of melatonin are in one gram of fresh bovine pineal glands. There are 5×10^{13} units in 1 gram of melatonin. A standard melatonin solution was prepared by mixing two grams of lyophilized, defatted bovine pineal powder with 10 ml. of water in a Waring blendor. The mixture was centrifuged at 16,000×g. for 30 minutes. The supernate was filtered and then extracted with an equal volume of redistilled ethyl acetate. The ethyl acetate was removed *in vacuo* in a flash evaporator at 40° C. Approximately 10 ml. of water was added to the residue, and the solution was kept on the flash evaporator at 40° C for 10 minutes to remove traces of ethyl acetate. The residue then was dissolved in water and made up to a volume of 100 ml., which was divided into 10 ml. portions and stored in the deep freeze. Each ml. contained approximately 30,000 units of melatonin. Care was taken throughout this work to avoid unnecessary exposure of melatonin solutions to light.

If a photometer unit is not available for use with a microscope, it is possible to determine the melanocyte (or melanophore) index (1) by studying five fields from each piece of skin at the times when photometer readings would be made. The minimum effective concentration is taken as the dilution necessary to produce a melanocyte index that is one less than that for starting skins.

RESULTS AND DISCUSSION

Frog skin was tested for ability to darken and lighten, and the ease of using skin from different anatomical areas was compared. These studies showed that the response was best for skin from the median-dorsal part of the thigh (Table 1). Technically, skin from this site was easy to work with. As mentioned previously, skin samples from the right and left legs of one frog gave a more similar response than specimens obtained from different frogs. Whereas unmatched skin samples were used for approximate assays, matched skin specimens were required for precise results. This is illustrated by the figures shown in Table 2 where the ratio standard error/mean value is used to characterize the precision of the estimates. The estimates in each

TABLE 1. COMPARISON OF SKIN SAMPLES FROM DIFFERENT REGIONS OF FROGS WITH REGARD TO THE RATIO OF MAXIMUM POSSIBLE DISPERSION M_d OF MELANOCYTE GRANULES TO MAXIMUM POSSIBLE AGGREGATION M_a

Skin area	$\frac{M_d^*}{M_a}$ Frog I	Frog II
1. Thigh: median dorsal	1.81	1.98
2. Thigh: dark area	1.72	1.71
3. Thigh: light area	1.33	1.35
4. Thigh: dark spots	2.83	2.67
5. Leg: dark area	1.58	1.96
6. Leg: light area	1.55	1.72
7. Web of foot**	1.12	1.16
8. Arm: dark area	1.30	1.75
9. Arm: light area	1.48	1.58
10. Back	1.04	1.02
11. Sides of trunk	1.40	1.96
12. (Tadpole skin)	2.75	2.40

* The mean values obtained from three different fields of each skin sample were used to calculate M_d/M_a.

** The melanocyte density in the web was very small.

TABLE 2. COEFFICIENTS OF VARIATION OF ASSAYS USING MATCHED AND UNMATCHED PIECES OF FROG SKIN

Experiment	Matched skin	Unmatched skin	
		1 specimen	2 specimens*
A	14.3	50.7	21.6
B	26.5	60.4	41.2
C	28.8	54.4	34.4

* These two samples were obtained from different frogs.

case were more precise, that is, had a lower coefficient of variation, when matched skin was used for the assay.

The dose response curve for melatonin showed a straight line relationship between Δ_1/Δ_2 and the logarithm of the concentration over a fairly wide range (Fig. 3). As shown in Figures 4 and 5, more reliable results were obtained from different batches of frogs if Δ_1/Δ_2 was plotted against concentration of melatonin than if the photometer readings were plotted against concentration. All these data show that the assay can be used with relatively little error.

Either transmitted or reflected light can be used for the bioassay. However, it is easier to work with transmitted light. Small changes in the water level greatly affected the photometer readings when light used is reflected but not when it is transmitted. Guanophores and xanthophores in skin contribute more to light reflectance than to its transmission so that for microscopic measurements it is better to use transmitted light.

At the end of the experiment it was important to check the ability of the skin to lighten with noradrenaline or melatonin. Occasionally substances toxic to the melanocytes, e.g., organic solvents, were present in the solution to be assayed so that the skin could not respond to the melatonin in the sample. Sometimes the assay procedure was prolonged over several hours because it might take a while to attain the proper dilution of the unknown. The melanocytes remained reactive during an 8-hour period and could be used without producing significant error.

The lightening produced by melatonin was usually of at least three hours duration. However, when small concentrations of melatonin were used, the skin became dark after 1 hour.

The bioassay can be made rather specific for melatonin. Noradrenaline, adrenaline, serotonin and acetylcholine, as well as melatonin, cause aggregation of pigment granules in melanocytes. All these substances reverse the darkening produced by ACTH or MSH, and all but acetylcholine reverse the darkening caused by caffeine. Thus if the skin was darkened originally by caffeine, acetylcholine would not interfere with the bioassay. The lightening produced by noradrenaline, adrenaline and serotonin is inhibited by ergotamine or related compounds; but the melatonin effect is not. If there were any question as to whether the lightening was due to noradrenaline, adrenaline or serotonin, as opposed to melatonin, ergota-

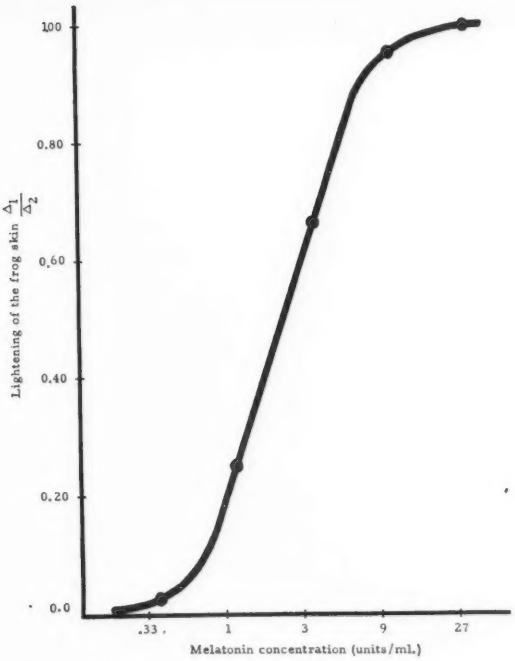

FIG. 3. The dose response curve for melatonin was obtained by plotting the mean values representing the readings from five fields for each of six pieces of frog skin.

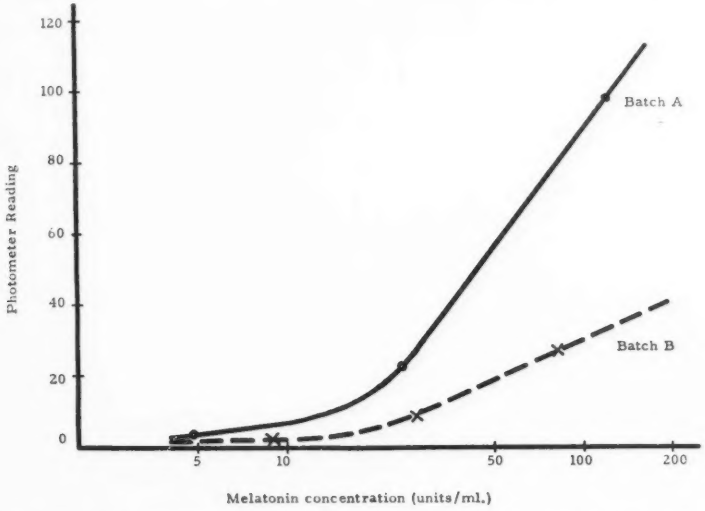

FIG. 4. Analyses were carried out on different days using two batches of frogs. Each curve represents the mean values from five fields for each of six skin samples. Photometer readings were plotted against concentration of melatonin.

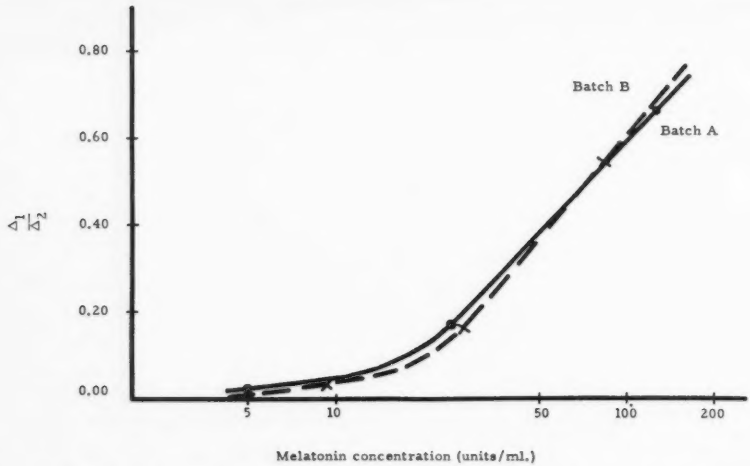

FIG. 5. The data from which Fig. 5 was drawn were replotted using Δ_1/Δ_2 instead of the individual photometer readings. Δ_1/Δ_2 values gave more consistent results than the photometer readings alone.

mine could be used in the test to distinguish melatonin from the other compounds.

In the bioassay we defined the minimum significant change in the color of frog skin as equal to 20% of the maximum possible lightening. However, lightening changes as small as 7% of the maximum lightening value were significant.

While this procedure was used primarily to test for melatonin, it could be modified in order to assay other lightening agents or darkening agents. The bioassay can detect 10^{-11} grams of melatonin. Physical methods, particularly fluorescence measurements, also could be used; however, they are less sensitive.

Acknowledgment

We want to thank Mr. David Skolnick for assisting in the early development of this assay procedure.

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INFLUENCE OF PROPYLTHIOURACIL ON THE THYROXINE-THYROTROPIN INTERPLAY[1]

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ABSTRACT

Normal and thyroidectomized rats were treated with propylthiouracil and thyroxine. The intact animals given 2 μg. thyroxine per day exhibited goiter and increased thyrotropin along with greater than normal protein-bound iodine in the serum. After thyroidectomy, 2 μg. thyroxine per day produced normal concentrations of both thyrotropin and protein-bound iodine; in similar animals treated with propylthiouracil thyrotropin was again normal but protein-bound iodine was twice normal. Propylthiouracil thus appeared to have an extra-thyroidal action on injected thyroxine as indicated by marked increase in protein-bound iodine.

THERE is mounting evidence for an extra-thyroidal effect of thiouracil and its derivatives on the metabolism of thyroxine in the rat. In the thyroidectomized animal (1) methylthiouracil was shown to lessen the increase in oxygen consumption produced by the injection of thyroxine (2) and a dose of thyroxine was found to have a greater effect on oxygen consumption in thyroidectomized rats than in those made similarly hypothyroid with thiouracil. Further, it was found that a greater proportion of the radioactivity of injected radiothyroxine was excreted in the feces in the presence of propylthiouracil (3, 4); if the I^{131} excreted in the urine in these circumstances was the inorganic residue from metabolized thyroxine, then propylthiouracil apparently slowed the degradation of thyroxine. Recently Van Middlesworth, Jagiello and VanderLaan (5) showed that the minimal dose of injected thyroxine required to prevent the production of goiter by propylthiouracil led to a protein-bound iodine concentration in the plasma which was approximately twice normal; goiter developed despite a protein-bound iodine concentration which was at least normal. As further investigation of these seemingly specific effects of thiouracil derivatives we measured thyrotropin and protein-bound iodine in the serum of rats treated with thyroxine and propylthiouracil and of others

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which had been thyroidectomized and treated with thyroxine with or without propylthiouracil.

METHODS

Male Sprague-Dawley rats obtained from the Charles River Laboratories of Boston were used throughout.

Experiments 1 (a) and (b). Rats averaging 150 gm. in weight at the start of the experiment were fed *ad libitum* a diet identical with Diet A of Van Middlesworth *et al.* (5) and divided into 5 groups of 4 or 5 animals. Four of the groups had propylthiouracil added to the diet in a concentration of 0.03% and 3 of the groups received, in addition, daily subcutaneous injections of sodium-L-thyroxine in doses of 1, 2 and 3 μg. per rat; the fifth

TABLE 1. THE INFLUENCE OF PROPYLTHIOURACIL ON THE EFFECT OF INJECTED THYROXINE ON PROTEIN-BOUND IODINE AND THYROTROPIN OF SERUM IN THYROIDECTOMIZED RATS

Group*	Initial body weight	Final body weight	Thyroxine dose, μg./100 gm. final body weight	Protein-bound iodine, μg./100 ml. serum	Thyrotropin assay
	gm.	gm.			
Normal (6)		216††	Nil	2.1(2.2, 2.0)†††	120 ± 25§
2A(6)		172	Nil	1.0(0.4, 1.6)	345 ± 87
2B(4)		163	0.61	1.0(0.4, 1.6)	239 ± 100
2C(5)	140 ± 14	193	1.04	2.4(1.6, 3.2)	127 ± 17
2D(4)		172	1.74	3.6(3.2, 4.0)	95 ± 17
2E(6)		168	1.19	2.9(2.6, 3.2)	97 ± 14
3A(8)		176	Nil	0.6(0.4, 0.8)	192 ± 74
3B(5)		155	Nil	0.4(0.2, 0.6)	233 ± 26
3C(6)	127 ± 11	181	0.55	1.9(1.8, 2.0)	231 ± 46
3D(8)		164	1.22	4.1(3.8, 4.4)	146 ± 29
3E(8)		155	1.94	5.8(5.6, 6.0)	98 ± 24

* All rats except in the normal group were thyroidectomized. Figures in parentheses indicate number of rats per group. Rats in Groups 2E and 3B to E had propylthiouracil (0.1%) in the diet.

†† Mean.

††† Average of two pools of serum (see methods) with separate values following in parentheses.

§ Mean ± standard deviation.

For interpretation of thyrotropin assay see "methods."

group had neither propylthiouracil nor thyroxine. Two such experiments were carried out; in the first (a) the rats were killed after 13 days and in the second (b) after 28 days. Immediately after death the rats were weighed, blood aspirated from the inferior vena cava, and the thyroid glands excised and weighed on a torsion balance. The serum from the rats of each group was pooled for estimation of protein-bound iodine and thyrotropin.

Experiment 2. Rats of an average weight of 140 gm. were thyroidectomized and divided into treatment groups as shown in Table 1; 6 rats were kept as a normal control group. Of the thyroidectomized animals Group 2A was given no treatment, Groups 2B, 2C and 2D were given a daily subcutaneous injection of 1, 2 and 3 μg. sodium-L-thyroxine respectively and Group 2E 2 μg. thyroxine daily with, in addition, propylthiouracil added to the diet in a concentration of 0.1%. The diet for experiments 2 and 3 was finely ground Ken-L-Biskit which had previously been found to contain approximately 250 γ. iodine per kg. (6). Treatment was started on the day of operation and continued for 13 days. Twenty-four hours after the last injection the animals were weighed and killed with chloroform; blood was obtained as in Experiment 1. The operation site was examined with a dissecting microscope for evidence of remaining thyroid tissue. When

thyroid tissue was found (on two occasions) or suspected (in 6 rats), the animals were not included in the series.

Experiment 3 (*See Table 1*). In this experiment rats of average weight 127 gm. were left for 6 days after thyroidectomy before treatment with propylthiouracil and thyroxine was started. They were in five groups; rats in Group 3A received neither thyroxine nor propylthiouracil, those in 3B, 3C, 3D and 3E had propylthiouracil added to the diet in a concentration of 0.1% and also in 3C, 3D and 3E they were injected daily with sodium-l-thyroxine, 1, 2 and 3 μg. per day respectively. Twenty-four hours after the last injection the rats were killed, weighed and bled. The operation site was inspected as in Experiment 2. In both this and the previous experiment two pools of serum were obtained for each group of rats for estimation of protein-bound iodine, half the animals contributing to the first pool and the remainder to the second.

Protein-bound iodine was measured in duplicate by a modification of the method of Zak *et al.* (7); in Experiments 2 and 3 these determinations were carried out at the Boston Medical Laboratory. Thyrotropin assays were carried out on serum pooled from all the rats of a group. The method was based upon an injection of thyrotropin causing an increase in circulating radioactivity in mice the thyroids of which had previously been labelled with radioiodine (6). The increase was expressed as a percentage of the radioactivity before injection; in control studies the value at 2 hours was always found to have fallen to about 97% of the initial radioactivity (6). The sera were assayed by the injection of 0.5 ml. intravenously into each of 5 mice. No standard preparation of thyrotropin was assayed concomitantly; however, a response of about 200 was usually found with the injection of 0.1 mu. U.S.P.

RESULTS

Experiment 1. The results of Experiment 1 (a) are shown in Figure 1. With a daily dose of 2 μg. of thyroxine (0.815 μg. per 100 gm. final body weight) goiter was found (mean thyroid weight = 16.1 mg./100 gm. body weight, standard deviation (S.D.) = 3.0; control mean 5.6 mg./100 gm., S.D. = 1.1) with the concentration of protein-bound iodine in the plasma, 6.4 μg./100 ml. being greater than in the control animals (3.3 μg./100 ml.). The concentration of thyrotropin also was greater than in the control group (Student's "t" test, $P < 0.05$). When the rats were treated with propylthiouracil and thyroxine for 28 days (Experiment 1 (b)) the findings were similar. Thyroid enlargement was found (18.6 mg./100 gm.; control 12.7 mg./100 gm.) even when the protein-bound iodine was 8.5 μg. per 100 ml. (control 2.5 μg. per 100 ml.) and thyrotropin also was of a greater concentration than in the untreated group ($P < 0.02$).

Experiment 2 (*Table 1*). In this experiment a daily injection of 2 μg. thyroxine (Group 2C) reduced the thyrotropin in the serum of the thyroidectomized rats to approximately normal and led to a normal concentration of protein-bound iodine. A similar dose of thyroxine to rats treated also with propylthiouracil (Group 2E) gave a protein-bound iodine but slightly higher and thyrotropin was no longer found. The rats of Group 2E did not gain weight (24.7 gm. ± 16.8, S.D.) as well as the rats in 3C (33.8 gm. ± 16.8, S.D.). The failure of these animals to gain normally in weight is not explained.

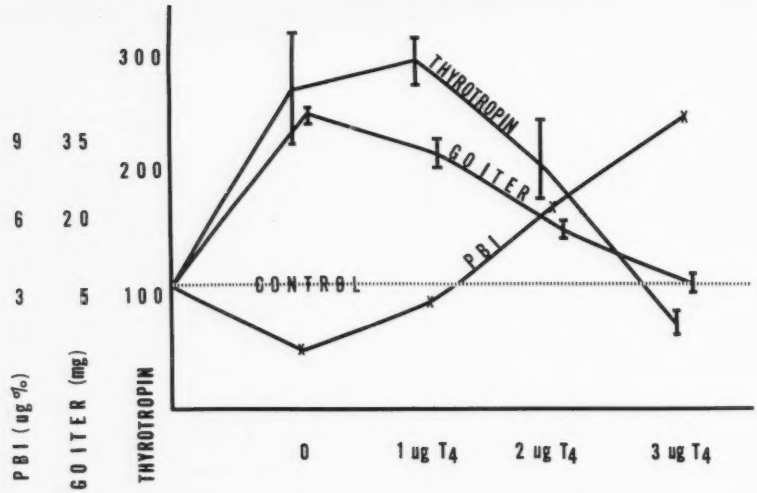

FIG. 1. *Assay of thyrotropin in goiter prevention test.* The dose of sodium-L-thyroxine (T4) given as a daily subcutaneous injection for 13 days is plotted on the abscissa. All except the control animals had propylthiouracil added to the diet in a concentration of 0.03%. The interrupted line impinging on the ordinate refers to the values in the control rats for protein-bound iodine (PBI) and thyrotropin of plasma, and the thyroid weight in mg. per 100 gm. body weight. The mean and 2 standard deviations are shown for the thyroid weight of each group.

The method of thyrotropin assay and interpretation of results are described under "methods"; the mean response and 2 standard errors of the mean are shown in each case.

Experiment 3 (*Table 1*). There was no significant difference in the thyrotropin concentration found in the serum of the thyroidectomized rats whether or not they were treated with propylthiouracil (Groups 3A and B). A dose of 2 μg. thyroxine per day (Group 3D) on this occasion led to a protein-bound iodine which was twice normal with, however, thyrotropin in approximately normal concentration. With 3 μg. per day (Group 3E), as in Experiments 1 and 2, thyrotropin was no longer found, but the protein-bound iodine was inordinately increased.

DISCUSSION

In Experiment 1 results similar to those of Van Middlesworth *et al.* (5) were obtained, but evidence was produced against their tentative explanation that goiter in those rats having a high concentration of protein-bound iodine in the plasma was due to an increased thyroid sensitivity to thyrotropin. It has been suggested by other workers (8, 9) that propylthiouracil enhances the action of thyrotropin on the thyroid. Our data do not exclude this possibility since we do not know whether, in the absence of the goitrogen, the concentration of thyrotropin found in the plasma of the rats in

Experiment 1 would have produced as much thyroid enlargement. Our findings, however, indicated that there was thyroid enlargement only when there was an increased concentration of thyrotropin in the plasma, and the same dose of thyroxine was required to suppress thyrotropin whether or not the rats were given propylthiouracil. Further, when propylthiouracil was given, the concentration of protein-bound iodine resulting from a dose of thyroxine was inordinately great.

Goldberg, Wolff and Greep (10) suggested that the metabolic action of circulating thyroid hormone, not the concentration, was the important factor in the regulation of thyrotropin output. It is of interest to compare our results with those obtained by Andik, Balogh and Donhoffer (1) and Barker, Kiely and Lipner (2). The former workers measured the oxygen consumption of thyroidectomized rats force-fed methylthiouracil for 4 weeks before injections of thyroxine for up to 4 days and found less of a response in these animals than in thyroidectomized rats not fed methylthiouracil. Barker *et al.* had similar results when they compared the increase in oxygen consumption produced by a single dose of thyroxine in thyroidectomized rats and in those made similarly hypothyroid by treatment with thiouracil. These experiments, especially the latter, were studies on the acute effect of injected thyroxine and are thus not entirely comparable with our results after chronic injection of thyroxine.

Furthermore, treatment with thiouracil derivatives might have a nonspecific toxic effect as evidenced by a lessened weight gain in the appropriate groups of our experiments, and it seems feasible that the oxygen consumption of rats could be adversely affected by such toxicity while a more specific effect of injected thyroxine, suppression of thyrotropin, was not. Griesbach and Purves (12) adduced from cytological changes in the pituitary that suppression of thyrotropin by injected thyroxine in thyroidectomized rats was uninfluenced by the addition of thiourea to the drinking water.

The mechanism of this extra-thyroidal action of propylthiouracil is not known but does not appear to be common to all goitrogens; goiter induced in rats by potassium perchlorate (4) and sulphadiazine (13) can be prevented by a dose of thyroxine which produced a normal protein-bound iodine. It is possibly related to the influence of propylthiouracil on the excretion of radioactivity by rats injected with radiothyroxine; both Van Arsdel and Williams (3) and Jones and Van Middlesworth (4) showed that under the influence of the goitrogen a greater proportion of this I^{131} appeared in the feces. If this represented a maintenance of the injected radioactivity in an organic state, then an increased concentration of protein-bound iodine might be expected from the injection of a given dose of thyroxine. A sequel to this hypothesis would be to question the specificity of the protein-bound iodine determination in these circumstances.

Another possible interpretation is that propylthiouracil has an effect on

the response of peripheral tissues, including the anterior pituitary, to thyroxine; in this case increased amounts of thyroid hormone, measured as protein-bound iodine, might be required to suppress thyrotropin secretion. However, this hypothesis would not of itself explain the increase in protein-bound iodine when the injected daily dose of thyroxine is constant.

Alternatively, it may be that propylthiouracil has an effect on thyroxine-binding globulin similar to the effect of estrogens which, in man, increase either the total amount or the capacity of that protein of plasma (14). If so, this could explain the finding of Hamolsky and his colleagues (15) that propylthiouracil given to patients with hyperthyroidism decreased the *in vitro* red blood cell uptake of I^{131} L-triiodothyronine, in some cases as early as 24 hours after the start of therapy; the red cell uptake of I^{131} L-triiodothyronine is apparently determined, in part, by the capacity of the thyroxine-binding globulin of the plasma for the radioactive hormone (15).

Certainly it is not possible from existing data to define clearly the specific mechanism which underlies our findings.

Acknowledgment

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EXCESS GROWTH IN TADPOLES AFTER TRANSPLANTATION OF THE ADENOHYPOPHYSIS[1]

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ABSTRACT

The primordium of the adenohypophysis was transplanted to the animal's own tail in frog (*Rana pipiens*) embryos. The growth of these animals was compared under standardized conditions with that of hypophysectomized, thyroidectomized and normal (operated and unoperated) controls. Growth in hypophysectomized animals was found to be retarded as compared to normals from the earliest feeding stages onward. Graft pituitaries were found to repair this growth deficit at all stages. At later tadpole stages a definite excess of growth rate over normals occurred in animals bearing displaced pituitaries. Thyroidectomized animals grew as normals in premetamorphic stages. Later they exceeded normals and, lacking metamorphosis, eventually reached giant size. Pituitary transplant animals also grew rapidly in postmetamorphic stages and attained giant size. An inhibitory control by the brain over the production of pituitary hormone(s) stimulating growth in the tadpole is postulated.

A MEASURE of control of some of the functions of the adenohypophysis by the brain is generally accepted today (1, 2, 3). Since secretory innervation appears to be absent, the pathway of hypothalamic control is commonly considered to be through the neurosecretory cells and the portal circulation (4, 5). In mammals ACTH, TSH, and gonadotropin production seem to be, at least in part, under control effected through the neurosecretory pathway. Confirmatory evidence in other vertebrates, particularly birds, exists and supports the concept that this mechanism is generally operative in vertebrates (6, 7).

Some years ago the senior author studied this problem by transplanting the primordium of the epithelial pituitary in the frog and analyzing some of the hormonally controlled factors in the animal's development. An excess of intermedin was found to be produced by graft pituitaries. From this the concept of an inhibitory control of the pars intermedia by the brain was developed and confirmed in various ways (8, 9). The metamorphic results obtained in the hosts indicated that some of the pituitary grafts produced thyrotropin, generally in subnormal quantities. It is the purpose of this paper to report new experiments that analyze the effects on body growth produced by autografted pituitaries in tadpoles.

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METHODS

Eggs of *Rana pipiens* were obtained by induced ovulation. Operations were performed on early tailbud embryos (stage 17). The primordium of the adenohypophysis is then a nubbin of cells projecting inward under the anterior end of the recently closed neural tube and still broadly attached to the deep layer of embryonic ectoderm from which it is derived. The tissue taken for transplantation included the nubbin, a disc of adherent ectoderm plus the attached endoderm of the foregut. The graft was inserted into a previously prepared pocket under the ectoderm of the same animal's tailbud.

Four types of animals were prepared. Type A was composed of unoperated and sham-operated controls. The latter were animals in which a slit was made in the pituitary region and in the tailbud but no tissue was removed or transplanted. Since both types of controls behaved alike as seen in their subsequent history, they will be reported as one. Type B was composed of hypophysectomized tadpoles operated at stage 17. A successful operation of this kind is recognized after about one week when the animal shows the silver condition due to absence of the pars intermedia. In previous experience of the senior author and other workers only very rarely does such an animal have an anterior lobe and show metamorphosis. None were found in the present study. Silver animals showing abnormality in the mouth parts or evidence of any inability to feed normally were discarded. Type C were the hypophysectomized hosts to pituitary autografts operated as described above. Type D was composed of thyroidectomized animal. Since this operation was done at stage 19 or 21 the handling of this group could not parallel that of the others in all respects. Animals of type B (hypophysectomy) which showed normal pigmentation upon development into tadpoles were regarded as having their normal pituitaries and were merged with the sham-operated controls. Type C (graft hosts) showing silver pigmentation (about 10%) were regarded as graft failures and were merged with the hypophysectomies. In all cases the subsequent history of these animals with respect to pigmentation and metamorphosis justified the classification. Successful thyroidectomies were recognized by failure of metamorphosis. Because of the unexpectedly high degree of success in the pituitary graft group (90%) the number of Type C animals was larger and the number of hypophysectomized animals smaller than had been intended. In all, the numbers of animals studied were 96 controls (Type A) 82 hypophysectomies (Type B), 102 animals with autoplastically transplanted pituitaries (Type C) and 13 thyroidectomies (Type D).

It is possible for a Type C animal to have a successful graft in its tail and yet have a pituitary also at the normal site since if even a small part of the pituitary area is left behind at operation a gland may develop by embryonic regulation. Type C animals might, therefore, have a pituitary at the graft site, at the normal position or at both. In the grown tadpole the infundibular region is readily examined by dissection under a stereoscopic microscope. It is then possible to determine satisfactorily the presence or absence of a gland at this site. This was done for all autografted animals as necessary. The presence of a gland at the graft site can be inferred by the pigmentation of the host and verified by serially sectioning the graft area of the tail.

In the experience of the senior author, the growth rate of *R. pipiens* tadpoles varies greatly from batch to batch despite standardization of conditions. For this reason, care was taken to do the operations and handling of the animals of differing experimental types in as parallel a manner as possible. For example, operations of each type (except thyroidectomy) were done at the same sitting using animals derived from the same batch and dish.

The tadpoles were kept under conditions which have been standardized in this laboratory as being close to optimal. Tadpoles begin to feed after stage 25 when the total length of the animal is 12–14 mm. At about this time the animals were set out individually in

four inch fingerbowls containing about 100 cc. of spring water and were fed canned spinach ad lib., care being taken that some food was present at all times. Our experience confirms that of Briggs and Davidson (11) that this diet allows maximal growth despite the coincidental formation of oxalate calculi in the kidneys. Animals were kept in a constant temperature cabinet at 23.5 ± .5° C. Water was changed three times weekly, except for very large animals over 70 mm., where daily change was necessary. Tadpoles attaining a length of 90 mm. were placed into five gallon fish tanks.

The total length and the hind leg length of the animals were measured, generally twice weekly, by transferring the animals to a flat dish over an illuminated millimeter scale against which the appropriate reading could be made with a minimum of handling or disturbance of the animal. An eyepiece micrometer was used for measuring legs less than 5 mm. The total length is considered the best and most convenient measure of tadpole size. Weight is unsatisfactory as it includes considerable variation in intestinal contents and requires harmful handling of the animals. Under standard conditions weight

TABLE 1. WEIGHTS OF TADPOLES SACRIFICED AT ABOUT 60 MM. TOTAL LENGTH

Type	*Group 1* Wt. gm. (No. animals)	*Group 3* Wt. gm. (No. animals)
A (Normal)	1.73 ± 0.13(16)	2.22 ± 0.67(4)
C (Graft Hosts)	1.72 ± 0.16(14)	2.05 ± 0.39(6)
B (Hypophysectomy)	1.44 ± 0.24(7)	

correlates well with the cube of length in normal animals (12). In the present experiment, a series of animals killed at 60 mm. total length were weighed before sacrifice. As Table 1 shows, normals and graft hosts at this length showed the same weights whereas the hypophysectomized animals were probably lighter as would be expected since they feed less. It is apparent, therefore, that if weight were used as the criterion of size, normals and graft animals would show the same relationship to each other and their superiority to the hypophysectomized animals would be greater than here reported.

As reported previously (8), graft animals tended to show excess pigmentation. Animals bearing autografts (Type C) were classified as showing excess intermedia activity when their pigmentation reached a level in excess of the maximum attained by normal animals kept in the light on dark backgrounds. Thyrotropin production by grafts was judged by progress of the hosts in respect to metamorphosis which is regarded as beginning with rapid hind leg growth (12). In the graft animals, hind leg length over 3 mm. at total length of 60 mm., or over 6 mm. in animals over 90 mm. was interpreted as evidence of at least minimal thyrotropin production since in thyroidectomized animals the legs were always smaller than these standards.

In the present experiment, a series of animals of each type was sacrified at 60 mm. total length for use in adrenal and other studies not reported here. Although these animals were selected in a way to avoid disturbance of the average growth rates of each type by matched selection from the same percentile levels, the growth rate date of this experiment are considered most reliable in the range up to 60 mm. (i.e. beginning of metamorphosis) and our interpretation will be based upon animals in this range.

Growth rates were determined in the following manner. A growth curve (length plotted against age) was constructed for each animal. To determine the growth rate of that animal at a particular length, say at 30 mm., the point of intersection of the growth curve with the 30 mm. ordinate was marked and the size of the animal three days before and three days after read from the graph. The difference between these readings is the amount of growth in the six days around the time the animal reached 30 mm. This was

taken as a measure of its growth rate at this time. The arrays of figures thus obtained for each type at the different sizes could then be treated by the usual statistical techniques. A measure of overall growth rate before metamorphosis was obtained by taking the total growth in the first twenty days of tadpole life.

The experiment was set up in triplicate, animals in each group being derived from a separate batch of eggs. Since the growth of controls differed significantly among the three groups, the growth rates for each of the different operative types (A, B, C and D) were compared with each other only within each group (1, 2 or 3).

RESULTS

Table 2 shows the growth in the first twenty days. It can be seen that in each of the three groups the hypophysectomized animals (Type B) grew at a significantly lower rate than normals. The differences are statistically clear except in Group 2 which had only seven control animals. In group 1 this difference amounts to 20.4% ($P < .001$). This is in agreement with

TABLE 2. GROWTH IN TWENTY DAYS AFTER EARLY FEEDING STAGE

Group	Starting length mm.	Growth mm. (No. animals)		
		A = Normals	B = Hypx	C = Graft Hosts
1	20	18.1 ± 0.6(70)	14.4 ± 0.9(30)*	24.6 ± 1.1(50)*
2	20	27.1 ± 4.3(7)	20.4 ± 1.0(23)	31.4 ± 2.9(12)
3	15	35.1 ± 2.6(15)	24.4 ± 1.2(23)*	36.7 ± 2.1(27)

* Difference from normal statistically significant $P < .001$.

the original investigations of Smith (13) and confirmed by many workers thereafter although, to our knowledge, no published quantitative studies are available.

The most striking fact shown by Table 2, however, is that the graft animals (Type C) grew as well or better than normals in each of the three sub-experiments. In group one the graft animals gained 35.9% more in length than their normal controls ($P < .001$). In each of the other two groups the difference is also in favor of the graft animals but it is smaller and not statistically significant. The growth of the graft animals, of course, exceeded that of hypophysectomized controls in each case by a clearly significant difference, amounting in each case to more than 50%. It should be noted that the time period selected for Table 2 represents most of the premetamorphic period of the animal's life and terminates before rapid hind leg growth begins.

A more detailed analysis of the data, one which exhibits the growth rates of the different types at successive points in the animal's development is given in Figure 1 for Group 1 and in Figure 2 for Group 3. Group 2 was omitted as having too few animals to yield statistically clear results, but it should be said that the data from this group are entirely consistent

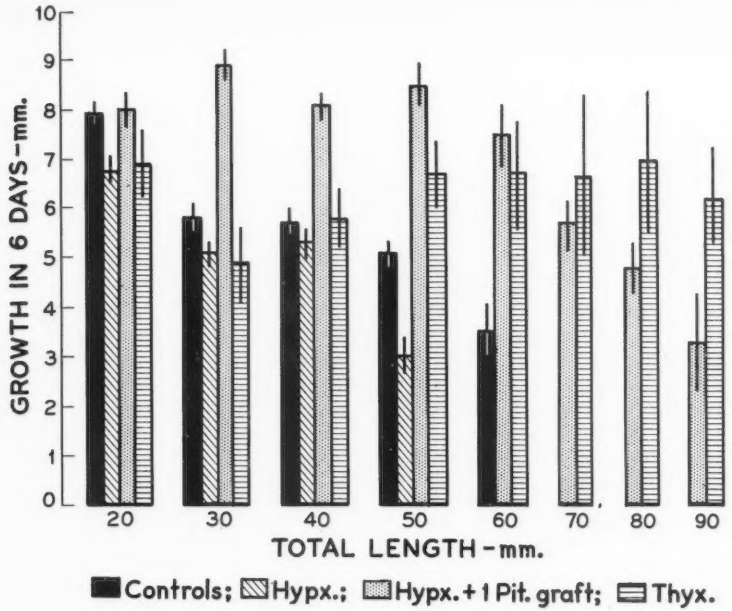

FIG. 1. Rate of growth of Group 1 tadpoles of different types at various stages of development (total length). Standard deviation of the mean shown by thin line.

with those of the two larger groups. Figure 3 illustrates a representative set of animals.

An examination of Figures 1 and 2 shows that, contrary to many statements in the literature (13), the difference between normal and hypophysectomized animals appears at the very outset of the tadpole period, i.e. in the six days around the 20 mm. stage. The growth rate of both normals and hypophysectomized animals decreases with increase in animal size, the hypophysectomized remaining below normals at all points, for the most part clearly and significantly so. The growth rate of hypophysectomized animals after 50 mm. falls so low one may legitimately speak of a growth stasis in most such animals. In neither group was there a sufficient number of hypophysectomized animals which reached 60 mm. size to warrant statistical treatment. The largest hypophysectomized animal reached a total length of 72 mm., the next 68 mm., but most never exceeded 65 mm. even when transferred to fish tanks in which graft and thyroidectomized animals often reached over 110 mm.

These diagrams show clearly that the production of the growth factor (presumably a hormone or combination of hormones) by grafts likewise begins in earliest tadpole life. In both group one and three there is a statistically clear difference between graft hosts and hypophysectomized

animals at each of the levels studied beginning at 20 mm. In comparing the graft hosts and normal animals, we find that the former grew more rapidly than normals at the 30, 40, 50 and 60 mm. levels in Group 1. In Group 3 a clear difference in favor of the host animals did not appear until the 50 mm. level (possibly also at the 40 mm. level, $P < .05$). The data suggest that the graft secretes the growth factor at least as rapidly as normal glands from the beginning of tadpole life and that in the latter part of the premetamorphic period it is more active than the normal gland.

The growth of the graft animals past the 65 mm. stage, at which time

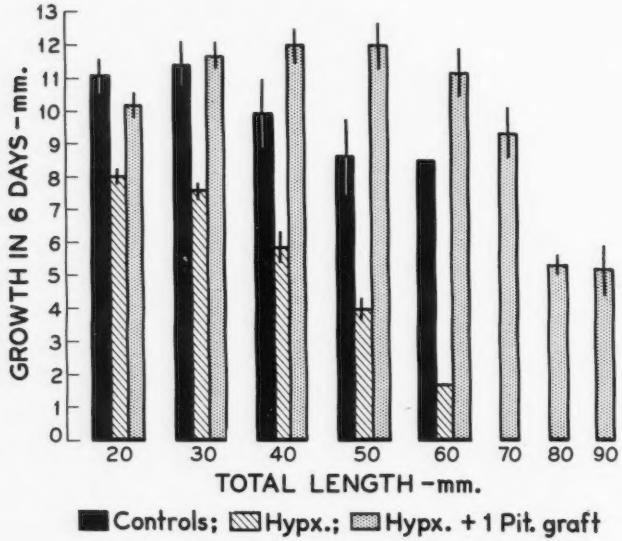

FIG. 2. Rate of growth of Group 3 tadpoles of different types at various stages of development. Standard deviations omitted where less than 10 animals available.

metamorphosis occurs in the controls, holds some interest although, as indicated under Methods, the data at this period are not strictly comparable to those of the earlier period. It is clear that the graft pituitary continues to produce growth factors as long as the experiment could be run. After 100 mm. the animals were difficult to keep alive, dying without apparent cause although some showed respiratory distress that suggested an imbalance between respiratory surface and needs. Such large animals, it should be noted, weighed eight to ten times as much as normals at the time of metamorphosis (Fig. 3). The decreasing rate of growth shown by these animals in the larger stages is probably real, but of course, not necessarily indicative of changing rate of production of growth factor by the graft.

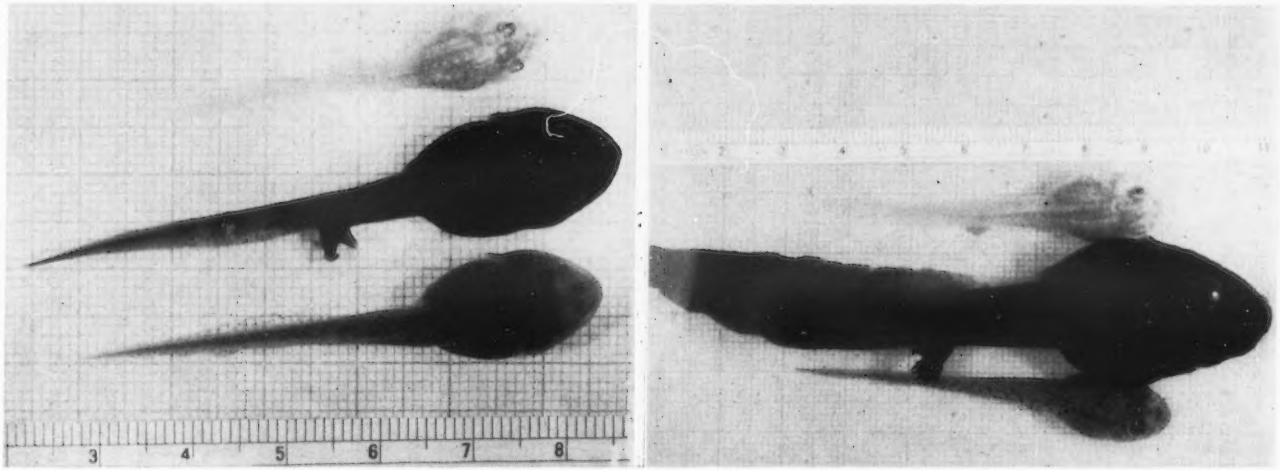

FIG. 3. Left. Normal (bottom), graft (center) and hypophysectomized (top) animals of Group 3. The protrusion on the side of the graft animal's tail is produced by mouth parts differentiated from the transplant. These animals belong to a matched set, i.e., each is derived from the same percentile level within its own type, the total lengths of the animals being 54, 63, and 41 mm. respectively. These are representatives of the differences in growth among the types in Group 3.

Right. Normal, graft and hypophysectomized animals not of a match set but illustrating the giant size attained by some graft animals (120 mm.) in contrast to the premetamorphic maximum (61 mm.) of a normal animal.

A set of 13 thyroidectomized animals was included in Group 1. It can be seen in Figure 1 that at least through the 40 mm. stage, the growth of these animals was very similar to that of the normals. Thereafter, with the approach of metamorphosis, they tended to exceed the normals since they did not show the marked decrease in growth rate characteristic of normals at this time. The difference between normals and thyroidectomized animals at the 60 mm. level approaches statistical significance ($P < .05$). Compared to the animals bearing pituitary grafts, the thyroidectomized are definitely slower at the 30 and 40 mm. levels, but not thereafter. It is noteworthy that in postmetamorphic sizes the thyroidectomized animals clearly maintain a high rate of growth though no statistical significance can be given to their differences from the graft animals. The data, as a whole, suggest that the pituitary of thyroidectomized animals secrete the growth factor more effectively than normal after reaching metamorphic size.

As explained under Methods, it is possible for the hosts of grafts to have pituitary tissue at both the graft and the normal site. It might therefore be thought that the excess growth of the autograft hosts might be due to the presence of excess pituitary tissue. A check on this possibility in the series of 26 animals which were sacrificed at the 60 mm. level showed six cases in which a normal-site gland was found. In each of these some anterior lobe tissue was also present in the graft area of the tail. An examination of the growth records of these six animals shows that none of them fell within the top 10% of their group. When their average growth at the 50 mm. level is compared with their group, it is clear that they fall somewhat below, rather than above, the mean. For example, the four individuals from Group 1 averaged 7.0 mm., as compared to the Group 1 average of 8.5. Details of the histological analysis of the pituitaries will be given in a later publication, but it may be said here that there is no reason to think that animals with pituitary tissue at two sites necessarily show more total anterior lobe tissue than those with the gland at only one site.

Of the autografted animals raised to maximal size, it was found that those which had pituitary tissue at the normal site were uniformly among the slower rather than among the fastest growing animals of their group. Thus, of the most rapidly growing 10% of all such animals (11 cases), none had epithelial pituitary at the normal site and must, therefore, have had pituitaries in the tail only. It is clear, therefore, that the superiority of the Type C animals over controls must be ascribed to the graft gland in the tail and not to excess tissue produced by embryonic regulation of the pituitary at the normal site.

A correlation between the production of growth factor and of other hormones might be expected on the basis that hormone production is a function of the viability of the graft. Our results, however, do not indicate any correlation with pigmentary hormone or TSH production. As indicated in Table 3, the growth rate of Type C animals is the same for those showing

TABLE 3. GROWTH RATE OF GRAFT HOSTS IN RELATION TO COLOR AND THYROID ACTIVITY
Amount of growth in mm. in six days at 40 mm. total length. (Number of animals)

	Group 1	*Group 3*
Nor. Pigmentation	7.82 ± 0.52(31)	12.34 ± 1.26(13)
Excess Pigmentation	8.44 ± 0.75(19)	11.82 ± 1.17(14)
TSH Activity	7.96 ± 0.65(24)	13.00 ± 1.09(14)
No TSH Activity	7.96 ± 0.57(24)	11.07 ± 1.25(13)

marked excess pigmentation and those showing pigmentation within the normal range. When graft hosts are classified into those showing some evidence of thyrotropin production and those showing no such hormone production, the growth rates in the two groups are again entirely similar.

DISCUSSION

The results detailed above show that the tadpole's pituitary is able to stimulate growth of the whole animal to a greater degree when transplanted to the tail than when left *in situ*. The question arises as to the nature of the growth effect, particularly, which of the pituitary hormones known from mammalian work may be involved. The inhibitory effect of hypophysectomy on the overall growth of an animal was first adequately described in the tadpole (13). This retardation is similar to that subsequently found in the rat (14). However, the later chemical work on pituitary extracts (15) which has led to the isolation of a purified pituitary factor that plays a central role in the growth effect and which has been called growth hormone or somatotropin has been carried out on mammalian test animals. To our knowledge, no published studies with purified growth hormone on tadpoles are available. A preliminary test which we ran with purified somatotropin, kindly supplied by Dr. I. Geschwind, failed to yield any growth stimulation at the dosage used. However, material for an adequate testing program was not available. In rats, pituitary grafts are only partially effective in restoring the growth rate of hypophysectomized hosts (16, 17). Lesions in the hypothalamus interfere with growth in the rat (18). If the growth factor demonstrated above in the tadpole is indeed somatotropin, then the manner of regulation of its release is presumably different in amphibians and mammals.

Prolactin production may be a factor in our results. This hormone probably occurs in the amphibian pituitary (19). Preparations rich in prolactin have been reported as especially effective in promoting body growth in pigeons, though not in rats (20). Most interesting in this connection are the reports that graft pituitaries in the rat maintain the secretion of the corpus luteum at a high level for long periods (21). If the growth factor with which we are concerned in the tadpole experiments is indeed prolactin, the analogy in the active production of the hormone in grafts in mammals and amphibia is striking. It is perhaps worth noting that synergistic effects often found with thyroid or thyrotropin appear to play no role here as no

correlation between the growth rate and metamorphosis was found. The possibility that ACTH is involved in the tadpole growth effect is suggested by reported effects of adrenal corticoids upon metamorphosis (22).

A second point meriting note is the unusual fact of excess hormone production by the graft pituitary. In mammals, hormone production of ACTH, TSH, STH and gonadotropins is thought to be subnormal or absent in grafts placed elsewhere than in the sella turcica (1). This is usually interpreted to indicate dependence of the production of these hormones upon stimulation by the hypothalamus in these animals. In contrast, the excess production of the growth factor in the tadpole suggests that this factor is under inhibitory control through the same connection to the brain. Inhibitory hypothalamic control was postulated for the pars intermedia in the tadpole (8, 9). The concept of inhibition by the brain may possibly apply also to prolactin secretion in the mammal. The only other instances of inhibitory influence of nerves on endocrine glands known to the authors are in invertebrates, namely the corpus allatum in some insects (23) and the optic glands of the octopus (24).

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THE EFFECTS OF THYROID AND PARATHYROID DEFICIENCY ON REPRODUCTION IN THE RAT[1]

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ABSTRACT

Sexually mature female rats were made thyroid-deficient by surgery or by irradiation of the thyroid gland with I^{131} or At^{211}. Thyroid hormone was replaced with desiccated thyroid, L-thyroxine, or L-triiodothyronine. Supplemental calcium was given orally as calcim gluconate. Animals were mated to test reproductive capacity 2 to 3 months after thyroid removal thus assuring complete elimination of residual thyroid hormone.

Thyroid-deficient rats had smaller litters and fewer pregnancies, only 31% compared to normal, 79% pregnancies. All three forms of thyroid hormone supplement increased the number of conceptions to 84%, and litter sizes were within control limits.

Stillbirths, prolonged gestation, and maternal tetany were common in I^{131}-treated groups as well as in surgically thyroparathyroidectomized rats regardless of whether or not supplemental thyroid hormone was given. In 46% of the cases tested the serum calcium level of I^{131}-treated, thyroxine-supported rats was less than 6 mg% at parturition, whereas the average serum calcium of normal controls was 10.5 mg%.

Variable radiation damage to the parathyroid glands of I^{131}-treated animals was observed histologically. There was a rough correlation between the extent of structural parathyroid damage and a lower than normal serum calcium level.

Thyroid hormone was shown to be essential for normal fertility and pregnancy, but its role in parturition, if any could not be demonstrated. The difficult labor observed in the I^{131}-treated rats was considered to be due to parathyroid deficiency.

INTRODUCTION

IT IS generally believed that reproduction is impaired in the thyroid-deficient female mammal. However, experimental investigation in laboratory rodents of the apparent relationships among the thyroid gland, the anterior pituitary, and the ovary has not yet led to firm conclusions. The extensive experimental literature on this subject has been reviewed by Peterson *et al.* (1).

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Disturbances in reproductive rhythm and structural alterations in the anterior pituitary and in the female reproductive organs are usually (but not invariably) found in chronically thyroid-deficient animals. The estrus cycle is prolonged in laboratory rodents following surgical thyroidectomy (2, 3) or pharmacological suppression of the gland (3, 4). Ross (2), Knud *et al.* (5), and Mazur (6) among others have reported a reduction in the number of maturing ovarian follicles in the hypothyroid rat. P'An (7) and Chu and You (8) reported ovarian and uterine atrophy in surgically thyroidectomized rats and rabbits.

Mating tests of thyroid-deficient laboratory rodents have produced the most contradictory results, and in a single species—the rat—observations range from sterility to no obvious reproductive impairment. The work of Ross (2), Barker (3), Chu (9), and Krohn and White (4) supports the view that hypothyroidism markedly interferes with reproduction in the female rat. Krichesky (10), Jones *et al.* (11), and Nelson and Tobin (12) found that litters were smaller, gestation was somewhat prolonged, and matings were occasionally sterile when rats were made thyroid-deficient surgically or with drugs shortly before or soon after conception. These latter investigators, however, did not regard their findings as clear-cut evidence for a specific effect of thyroid hormone on reproduction.

Watts (13) and Gemmill *et al.* (14), using the metabolic rate as an indicator, found that a severe hypothyroid condition does not exist in the rat until at least 40 days after thyroid gland removal. The time may be even longer between thyroid removal and the appearance of the histological and presumably functional anterior-pituitary changes associated with thyroid deficiency. The normal gestation period of the rat is 21 days. Therefore, a rat thyroidectomized or fed antithyroid drugs shortly before conception or early in pregnancy will have some residual thyroid hormone in its tissues during the gestation period. The presence of residual thyroid hormone with its supportive action on the ovary and pituitary gland may partially explain the inability of some investigators (10–12, 15) to demonstrate the marked reproductive impairment which is seen in animals with long-standing hypothyroidism.

Calcium and—by inference—the parathyroid glands are also important factors in successful reproduction. Mazur (6) and Bodansky and his co-workers (16–19) concluded from their studies of the surgically thyroparathyroidectomized rat that parathyroid deficiency was the main source of such difficulties at term as prolongation of gestation, stillbirths, and maternal tetany. They found that tetany could be prevented and normal parturition promoted by feeding a high calcium diet or by injection of calcium salts near term.

These experiments were designed to test the apparently separate influences of the thyroid secretions on fertility and fetal maturation and la-

bility and of the parathyroid secretions on parturition and maternal survival.

METHODS

The animals used were female rats of the Sprague-Dawley strain obtained from the parent colony when 70 days old. They were housed on wood shavings in metal cages, and were fed Purina Laboratory Chow[3] and tap water *ad lib.* The animals were sexually mature (22) when introduced into the experiment (75 days old in Experiment I and 110 days old in Experiments II and III). In each of three experiments 10 rats were set aside as controls. The remaining animals were thyroidectomized surgically or by irradiation with I^{131} or At^{211}.[4] Surgical thyroidectomy was carried out according to the method of Farris and Griffith (22). Radioiodine[5] was administered by intravenous injection at a level of 5 μC per gram body weight. This amount of I^{131} produces severe hypothyroidism with minimal structural damage to other organs (13). The At^{211} was prepared by methods described elsewhere (23) and was administered intravenously in isotonic saline at a level of 0.62 μC per gram body weight. This At^{211} dosage was found to produce maximal thyroid destruction with few acute radiation deaths (21).

Three essentially independent experiments were carried out. The experimental design, the ages of the animals, mode of treatment, and group sizes are shown in Table 1.

Experiment I

Ten days after thyroidectomy one-half of each group was placed on a supplement of desiccated thyroid (Armour). A freshly prepared aqueous suspension of the thyroid substance (TH) was administered intragastrically three times weekly. The surgically thyroidectomized rats received 9 mg./week of active thyroid substance, and the I^{131} and At^{211}-treated rats received 12 mg./week. Standard metabolic rates (SMR) of the animals in each group were determined by measuring the time required for the consumption of a measured volume of oxygen in a closed system. The apparatus and calculations employed were essentially those described by Kleiber (24), Contopoulos *et al.* (25) and Evans *et al.* (26) as modified by Watts (13). Before any measurements were made, the animals were conditioned to the apparatus twice daily for 30 minutes for a period of 2 weeks. Repeated observations were made over a period of 3 months or until two successive observations for individual animals agreed within 5%.

When the animals were 160 days of age, breeding experiments were initiated. The animals were mated by exposing each cage of 5 female rats to 14 different males over a period of two weeks. At the end of this period the females were placed in individual cages. As the animals neared term, the cages were examined several times each day. A record was kept of the number of young born alive or dead.

Eleven months after the start of the experiment, all animals were given a tracer dose of 20 μC of I^{131} intramuscularly. Twenty-four hours later the animals were sacrified with chloroform and the tracheas removed. The gamma-ray activity of the I^{131} in the residual peritracheal tissue was counted in a well-type NaI-TlI scintillating crystal counter.

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[3] Purina lab. chow contains 1.35% calcium by weight derived from skim milk, fish meal, and cereals. The Ca/P ratio is approximately 1.4 by weight.

[4] At^{211} is a radioactive isotope of the heaviest halogen. It emits alpha particles with a mean energy of 6.8 Mev and decays with a half life of 7.5 hours. The thyroid gland selectively accumulates At^{211}, and the intense alpha-particle irradiation is capable of destroying sufficient thyroid tissue to produce permanent hypothyroidism (20, 21).

[5] Oak Ridge National Laboratory carrier-free processed I^{131}.

TABLE 1. GROUP SIZE, MODE OF THYROID DESTRUCTION, AGE AT THYROIDECTOMY, AGE AT BREEDING, AND EXPERIMENTAL TREATMENT. (TH = DESICCATED THYROID SUBSTANCE, T4 = L-thyroxine, T3 = triiodothyronine)

Mode of thyroidectomy	Day of age at thyroidectomy	Treatment	Group Size	Days of age at breeding
Experiment I				
Surgery	75		13	160
Surgery	75	TH[a]	12	160
I^{131}	75		13	160
I^{131}	75	TH	12	160
At^{211}	75		10	160
At^{211}	75	TH	12	160
Controls	75		10	160
Experiment II				
I^{131}	110		11	220
I^{131}	110	Calcium[b]	12	220
I^{131}	110	T4	10	220
I^{131}	110	T4+calcium	10	220
Controls	110	Saline	9	220
Experiment III				
I^{131}	110	T4	10	200
I^{131}	110	T4+calcium[c]	8	200
I^{131}	110	T4+T3	9	200
I^{131}	110	T3	9	200
Controls	110		10	200

[a] Desiccated thyroid substance (Armour).
[b] Veterinary calcium gluconate by intubation daily for last 5 days of pregnancy.
[c] Calcium gluconate, C.P., by intubation daily for last 5 days of pregnancy.

The ovaries and peritracheal tissue were dissected, fixed in Bouin's fluid, and prepared for microscopic examination.

Experiment II

All the animals were thyroidectomized with I^{131}. Five days later 20 of the 45 I^{131}-treated rats were placed on a schedule of daily subcutaneous injections of 0.1 cc. of an alkaline solution of 60 μg./cc. Na-L-thyroxine (Smith, Kline & French Laboratories). This dosage of L-thyroxine (T4) has been found to sustain a normal SMR in the thyroidectomized rat (26, 27). This more potent form of thyroid hormone was administered parenterally in this and the subsequent experiment. Hormone dosage could be more closely controlled (than when a slurry was given by mouth), and irritation of the upper respiratory tract and aspiration of intubated fluids were avoided. One-half the thyroxine-treated animals, and half of the remaining thyroidectomized animals received 1.5 grains of veterinary calcium gluconate (Haver-Lockhart Laboratories) intragastrically once each day during the last 5 days of pregnancy. The 10 intact control animals received daily sham injections of 0.1 cc. isotonic saline subcutaneously. Beginning at 100 days post-thyroidectomy vaginal smears were examined to determine the estrus cycle (28). As epithelial cells appeared an adult male was placed in the cage with the female. The next morning the appearance of motile sperm or a sperm plug indicated conception had probably occurred. This was considered Day 1 of pregnancy (29). Sixty days after the last litter was delivered, the animals were autopsied as described above. The completeness of thyroidectomy was determined using a tracer dose of I^{131} as described in Experiment I.

Experiment III

Five groups of 10 rats each were used. Forty were given thyroid-destructive doses of I^{131}. Similar metabolic effects of L-thyroxine (T4) and L-triiodothyronine (T3) have been

demonstrated repeatedly (26, 30–34). However, it was considered possible that these two hormones might have some as yet unidentified independent actions and that either alone could not constitute adequate thyroid replacement during the many phases of reproduction. In this experiment T4, T3, and a mixture of the two hormones, T3 and T4, were used as supportive therapy. Thyroxine was given daily at a level of 7 μg. per rat (26). Triiodothyronine was administered daily at a level of 1.2 μg. per rat, an amount reported to sustain a normal SMR in the thyroidectomized rat (26, 35–37). A third group received a daily injection of a mixture of 6.3 μg. T4 and 0.12 μg T3—the proportions used were those found by Taurog *et al.* (38) in the thyroid vein of the sheep. A fourth group on the T4 regimen was given 65 mg. calcium gluconate (C.P.) daily by gastric intubation during the last 5 days of pregnancy. After 60 days of hormone administration, metabolic rates were determined.

The animals were bred by placing a fertile male in the cage with a female for seven consecutive days. Lack of space restricted matings to 20 at a time. The females were examined daily for indications of pregnancy and later, onset of labor. The (T3) and the (T4·T3) groups which were the first to be mated showed a high incidence of maternal tetany and stillbirths. These observations suggested a parathyroid deficiency evident only at term. Therefore, the remaining three groups of animals were sacrificed during labor or immediately following delivery for serum calcium measurement and parathyroid histology. With the animal under ether anaesthesia, blood was aspirated from the inferior vena cava into a heparinized syringe. Serum calcium was determined by the method of Baron and Bell (39). Peritracheal remnants were prepared for microscopic examination.

RESULTS

Thyroid destruction (in Experiment 1) with either of the radioactive isotopes, I^{131} or At^{211}, caused a reduction of the SMR to 65% of the control mean, as shown in Table 2. Watts (13) obtained similar results. Surgical removal of the thyroid gland resulted in an SMR only 15% below normal. Although the mean SMR of the surgically thyroidectomized group was within one standard deviation of the control mean, the t-test of Fisher (40) indicated that statistically, the two means were significantly different ($P < 0.01$).

The I^{131} tracer given 24 hours before sacrifice showed that surgical removal of the thyroid could be considered complete in five of nine cases;[6] the I^{131} uptake in peritracheal tissue was less than 0.1%. Radioiodine uptakes of the other four animals ranged from 0.8% to 2.2% of the administered tracer. There was, however, no detectable correlation between an uptake of the I^{131} tracer greater than 0.1% (indicating the presence of functional thyroid tissue) and a higher-than-average SMR or better-than-average reproductive capacity.

Thyroid destruction with I^{131} was judged complete in all cases; uptake of the I^{131} tracer ranged from 0.004% to 0.05% in Experiments I and II. Astatine, as reported previously, was not as efficient an agent for thyroid destruction as I^{131} (21). Uptake of the I^{131} tracer by the At^{211}-irradiated

[6] Four animals were lost to autopsy; one animal died on labor and three others died of pneumonia.

rats ranged from 0.01% to 1.4% of the dose. As in the surgically thyroidectomized group, there was no correlation between a higher uptake of the I^{131} tracer and an above-average SMR.

Desiccated thyroid substance at a level of 9 to 12 mg/wk brought the SMR of the surgically thyroidectomized animals and the At^{211}-irradiated animals up to low normal. However, 12 mg/wk of thyroid substance was

TABLE 2. THE EFFECT OF THYROPARATHYROID DEFICIENCY ON THE NUMBER OF SUCCESSFUL CONCEPTIONS IN THE RAT, AND SURVIVAL OF YOUNG AND MOTHERS; THE BENEFICIAL EFFECT IN THESE ANIMALS OF THYROID AND (OR) CALCIUM THERAPY

Group treatment	S.M.R. ±Std. Dev. Cal/m²/hr.	No. of Pregnancies	Avg. No. Young born alive[c]	Avg. No. Young born dead[d]	Mothers dying during labor[e]
Experiment I					
Surgery	44.0±5.3	7/13	4.3	3.0	1
Surgery (TH)	47.8±5.2	10/12	3.1	3.0	0
I^{131}	33.8±5.4	3/13	2.7	1.0	0
I^{131} (TH)	35.4±4.0	8/12	2.2	4.0	2
At^{211}	33.4±5.3	0/10	—	—	0
At^{211} (TH)	45.2±4.4	0/12	—	—	0
Controls	51.6±3.8	7/10	7.4	0.03	0
Experiment II					
I^{131}	Not run	1/11	6.0	0.0	0
I^{131} (Ca)[a]	Not run	4/12	3.0	3.2	0
I^{131} (T4)	Not run	9/10	1.4	5.1	2
I^{131} (T4·Ca)[a]	Not run	6/10	11.2	0.0	1[f]
Controls	Not run	7/9	not recorded		0
Experiment III					
I^{131} (T4)	41.4±4.5	10/10	10.3	0.1	4
I^{131} (T4·Ca)[b]	41.4±4.5	7/8	8.8	2.0	4
I^{131} (T4·T3)	40.3±5.6	8/9	7.7	2.7	3
I^{131} (T3)	34.4±3.0	9/9	6.7	3.3	5
Controls	36.3±5.9	9/10	10.6	0.1	0

[a] Veterinary calcium gluconate by intubation daily for last 5 days of pregnancy.
[b] Calcium gluconate (C.P.) by intubation daily for last 5 days of pregnancy.
[c] Includes young delivered alive and young found alive in utero at necropsy.
[d] Includes young born dead and found dead in utero at necropsy.
[e] Includes animals in tetany sacrificed for serum calcium measurements.
[f] In tetany after only 2 days treatment with calcium.

not sufficient to bring the SMR of the I^{131}-irradiated animals even to the lower limit of normal. Evans *et al.* (41) points out that while 1 μg. of L-thyroxine per day is not enough to maintain a normal SMR in the female rat, it is sufficient to promote normal skeletal growth and sexual development. The thyroid therapy was therefore considered adequate for the purpose of these experiments.

In Experiment III the use of T4 (45.5 μg/wk), T3 (8.4 μg/wk), or a mixture of 90% T4 and 10% T3 as thyroid replacement brought the metabolic rates well within the control limits. SMR's were not measured in Experiment II. The T4 treatment was similar in Experiments II and III, therefore it seems reasonable to assume that the animals in Experiment II re-

eived adequate thyroid hormone replacement. We have no ready explanation for the discrepancy between the two values shown for normal SMR. The results for each experimental group were reproducible and internally consistent. Watts (13) in this laboratory obtained a mean SMR for normal male rats of this strain and age of 43 ± 3 Cal/m²/hr, almost midway between the control means for Experiment I and II.

Surgical thyroidectomy led to a reduction in the number of pregnancies; only 54% of the matings resulted in conception. In the I^{131}-irradiated groups, in Experiments I and II the highest percentage of pregnancies was 33%, and the lower limit was less than 10%. No pregnancies were observed in the At^{211}-treated group. The ovaries of this latter group of animals were atrophic on histological examination and had apparently been severely irradiated.

The reproductive capacity of all thyroid-deficient animals (except those whose thyroid tissue had been destroyed with At^{211}) was greatly enhanced by giving them some form of thyroid hormone. The number of pregnancies was increased from 31% in the thyroid-deficient animals (15 pregnant of 49) to 84% in thyroidectomized animals that received some form of supplemental thyroid hormone (67 pregnant of 80). In the three control groups 23 of 29 rats or 79% became pregnant after a similar single mating. The radiation damage in the ovaries of the At^{211}-treated rats was apparently not repaired to any significant extent by thyroid therapy.

As shown in Table 2, the thyroid-deficient animals bore smaller litters—4 to 7 pups—than did the normal controls—7 to 11 pups. Stillbirths were rare in control litters, but were common in the litters of animals whose thyroids had been removed surgically or with I^{131}, regardless of whether or not supplemental thyroid hormone was given. The number of stillbirths occasionally exceeded 50% of the litter.

When thyroid therapy was employed in Experiments I and II, the litters were slightly larger, usually 6 or more pups. In Experiment III litters were of normal size, averaging 10 pups. This larger average litter size may be due to increased amounts (or more potent forms) of thyroid hormone, to more frequent hormone administration (daily rather than three times a week), or to more precise observations, or to a combination of these factors.

The high proportion of surviving young in Experiment III is harder to explain, and may be somewhat misleading. As mentioned above, the thyroid supplement had no apparent effect on the proportion of stillbirths. From 30% to 75% of the pups born to animals whose thyroid tissue had been removed surgically or with I^{131} were delivered dead, died within a few minutes after delivery, or were found dead *in utero*. The mothers in Experiment III were under almost constant observation and most of them were killed just before or during delivery. A number of live pups was generally found *in utero*. Had these mothers, particularly those in tetany, not been watched so closely many of their young might have died during or after

delivery, or died *in utero*. In such cases they would have been recorded as stillbirths.

The length of gestation was observed accurately only in Experiment II. Day 1 of pregnancy was established by the method of Blandau *et al.* (29). The gestation period of the control animals averaged 23 days (range 21–23 days), I^{131} (T4) averaged 24 days (range 22 to 26 days), I^{131} (T4·Ca) all delivered on Day 23, I^{131} (Ca) averaged 23 days (22 to 24 days), and I^{131}, one animal only, delivered at 23 days.

By combining the information from all experiments, it was found that 11 of 37 untreated thyroid-deficient animals (no thyroid hormone or calcium) became pregnant (30%); one died in labor. Fifty-four of 82 rats that were given thyroid hormone (but no calcium) became pregnant (87%), and 16 of these animals (30%) either died in labor or were in tetany when sacrificed. No deaths during labor were recorded for the 23 control pregnancies.

In Experiments II and III, 17 pregnant rats were given either veterinary calcium gluconate or calcium gluconate (C.P.) by mouth daily during the last 5 days of pregnancy. Five of these animals (30%) died in labor or were in tetany when sacrificed. One animal included in the data had received calcium gluconate for only 2 days when labor began. Oral administration of either veterinary or calcium gluconate (C.P.) did not seem to improve maternal survival. There was, however, some indication that the veterinary preparation had some beneficial effect on length of gestation and the viability of the young. In Experiment II the six animals that received both thyroid hormone and the calcium supplement delivered large litters without incident, and no stillbirths were found. All six litters were successfully suckled until weaning. On the other hand, half of the young born to four thyroid-deficient rats—I^{131} (Ca)—were delivered dead. In Experiment III, maternal survival was no better, and the percentage of live young born to the group that received calcium gluconate (C.P.) was only slightly better than for those groups that did not receive supplemental calcium.

In the third experimental series it was necessary, because of the large number of animals involved, to breed 20 animals at a time. Although the number of pregnancies was larger and the litter sizes were close to normal for the (T3) and (T4·T3) groups, it was obvious that these animals were delivering their litters with difficulty. On the average, one-third of the young were delivered dead, and 8 of the 17 pregnant rats in these two groups died in labor. The histological specimens from the two previous experiments were completed at about this time. In some of the sections of peritracheal tissues taken from I^{131}-irradiated animals, the parathyroid tissue, while still grossly intact, showed some indications of structural alteration. The structural changes of the parathyroid are discussed later in detail. Other investigators have commented on the absence of damage or have noted minimal damage of the parathyroid glands of rats whose th-

roid glands had been destroyed with minimal doses of I^{131} (2, 21, 42, 43). We had previously reported nearly normal serum calcium levels in rats given thyroidectomizing doses of I^{131} (44). These previous observations apply only to animals under little stress. In the experiments reported herein it was considered likely that termination of pregnancy might produce sufficient stress to disclose a previously masked partial parathyroid impairment. The remaining experimental and control rats were bred and

TABLE 3. SERUM CALCIUM LEVELS IN IODINE-131 THYROID-ABLATED RATS AT TERM

Treatment and animal number	Condition of mother at necropsy	Serum calcium level mg.%
Control		
2330	In normal labor	11.08
2342	Post-partum	9.95
2328	Delivering normally	10.16
2344	Post-partum	10.08
2333	Post-partum	11.82
2334	Post-partum	12.28
2353	In normal labor	10.20
2339	Delivering normally	10.70
2367	Post-partum	10.83
2329	Post-partum	10.67
(T4)		
2338	Delivering normally	8.74
2358	In normal labor	9.91
2337	Post-partum	10.49
2335	In tetany	3.89
2345	In normal labor	7.11
2363	Post-partum	10.24
2364	In tetany	5.16
2348	In tetany	3.83
2359	In tetany	3.12
(T4·Ca)		
2366	In normal labor	9.12
2369	In normal labor	8.85
2346	In tetany	4.08
2365	In tetany	4.46

observed with great care so that serum calcium might be measured during or very soon after labor. Serum calcium levels and the stage of labor when the blood samples were taken are shown in Table 3 for 10 normal controls, 9 animals in the I^{131}(T4) group and 4 animals in the I^{131}(T4·Ca) group.[7] Animals are designated in the Table as "in normal labor"—contractions and(or) vaginal bleeding but no pups yet born; "delivering normally"—one pup had been delivered, but the mother was sacrificed before the placenta was eaten; or "post partum"—some or all of the pups were delivered and placentae eaten. Those animals showing convulsions or rigidity are designated as "in tetany." The range of control values was 9.95 mg% to 12.28 mg. of calcium per 100 ml. of serum. The range for the experi-

[7] Four rats in these two groups either had died during labor or had successfully delivered their litters before serum samples could be obtained.

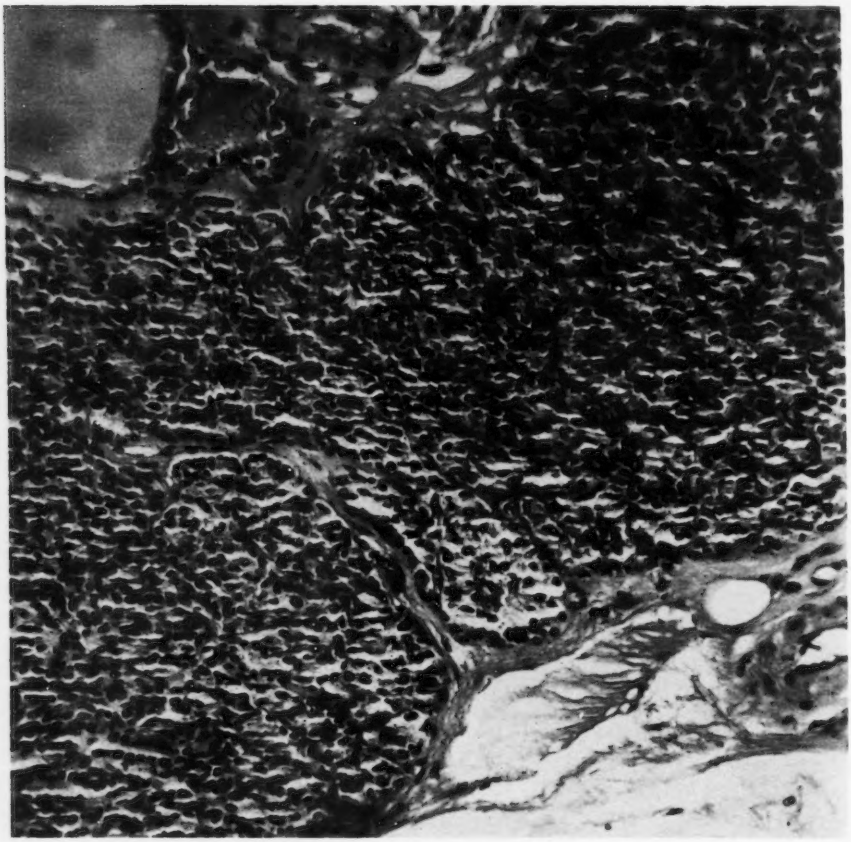

FIG. 1. Normal parathyroid tissue taken from a control animal in Experiment I. Note capsule of loose connective tissue and delicate reticular network. Cells in central region are arranged in cords; those at the periphery are arranged in clumps. Sectioned at 6 μ, H and E $\times$220.

mental animals was 7.11 mg.% to 10.49 mg.%, and for those in tetany the range was 3.12 mg.% to 5.16 mg.%.

Peritracheal tissue for histology was removed with the aid of dissecting microscope from the thyroid region of 18 I^{131}-irradiated animals in Experiment I, and from 15 I^{131}-irradiated rats in Experiment III for which a pre-autopsy serum calcium measurement was available. Both parathyroids were identified in only 8 of the 33 specimens examined; one gland was seen in an additional 17. The normal parathyroid gland as shown in Figure 1 is highly vascular and densely cellular, and the cells are arranged in irregular cords and clumps supported by delicate reticular fibers. The gland is covered by a delicate connective-tissue capsule.

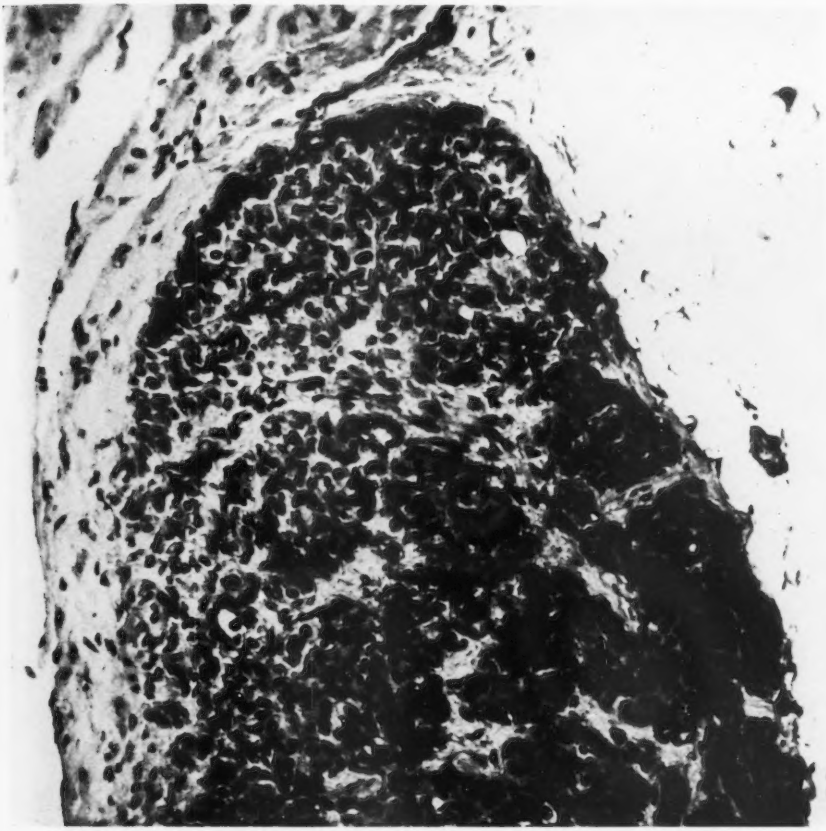

FIG. 2. Parathyroid from rat No. 2337; serum calcium 10.49 mg.% during normal delivery. A thick fibrous replacement of the thyroid. Note the conspicuous reticulum and reduced cellularity in the region nearest the trachea. This gland had apparently been located on surface of thyroid. Sectioned at 6 μ, H and E $\times$220.

None of the parathyroid glands taken from I^{131}-irradiated rats could, upon careful examination, be classed as undamaged. In general, these glands were smaller than those of normal controls. They were surrounded by a thick band of fibrous connective tissue which was usually continuous with the scar tissue that had replaced the thyroid (Fig. 2). When the parathyroid appeared to have been originally located on the surface of the thyroid, the connective-tissue capsule was thinner on the outer surface. Only rarely were blood vessels seen to be penetrating this capsule. The reticular network of the irradiated glands was conspicuous in many specimens and comprised a large proportion of the total tissue in some of them. The capillary network was generally very much reduced or obscured by

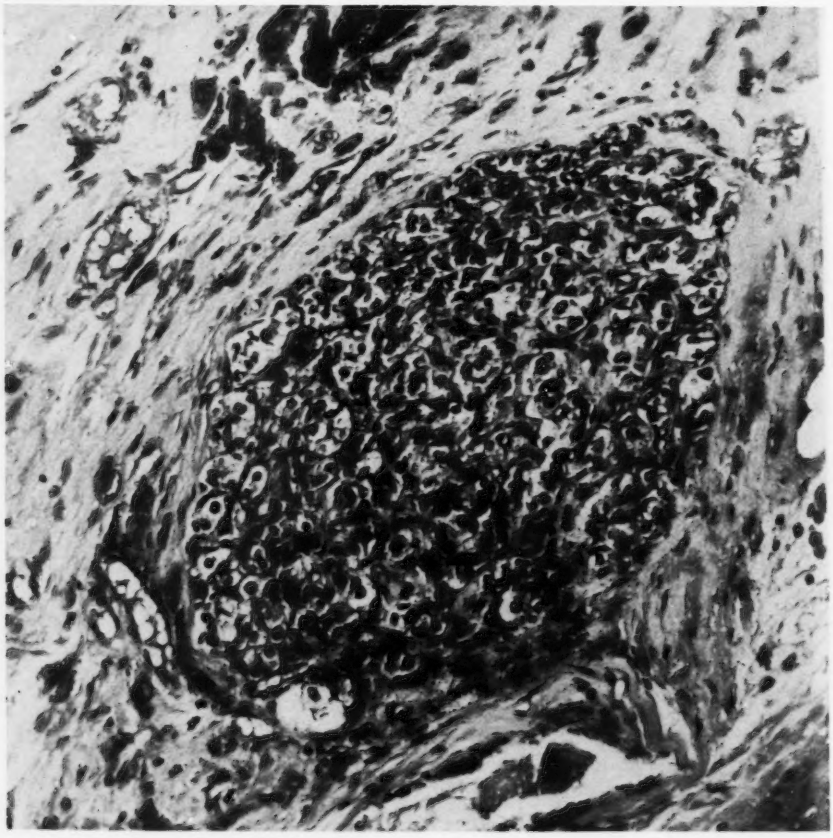

FIG. 3. Parathyroid from rat No. 2348; serum calcium 3.83 mg.%. Animal in tetany attempting delivery. Note thick fibrous capsule, and small clusters of cells separated from each other by connective tissue septa. This gland apparently has been embedded deep within the thyroid tissue. Sectioned at 6 μ, H and E $\times$220.

the connective tissue. Occasionally two or three large blood vessels were seen in a single section. The cellularity of the irradiated parathyroid glands varied from a few cells arranged in "nests" of two to four cells separated from one another by connective-tissue septa (Fig. 3) to densely cellular, deeply staining tissue with a structure more reminiscent of normal parathyroid (Fig. 4).

Correlation of structure with function was not clean-cut, particularly since only one parathyroid was usually seen. The two tiny pieces of peritracheal tissue from each animal were embedded in the same paraffin block. The blocks were not sectioned serially but were cut only until two pieces of tissue could be seen in the paraffin ribbon Consequently, identi-

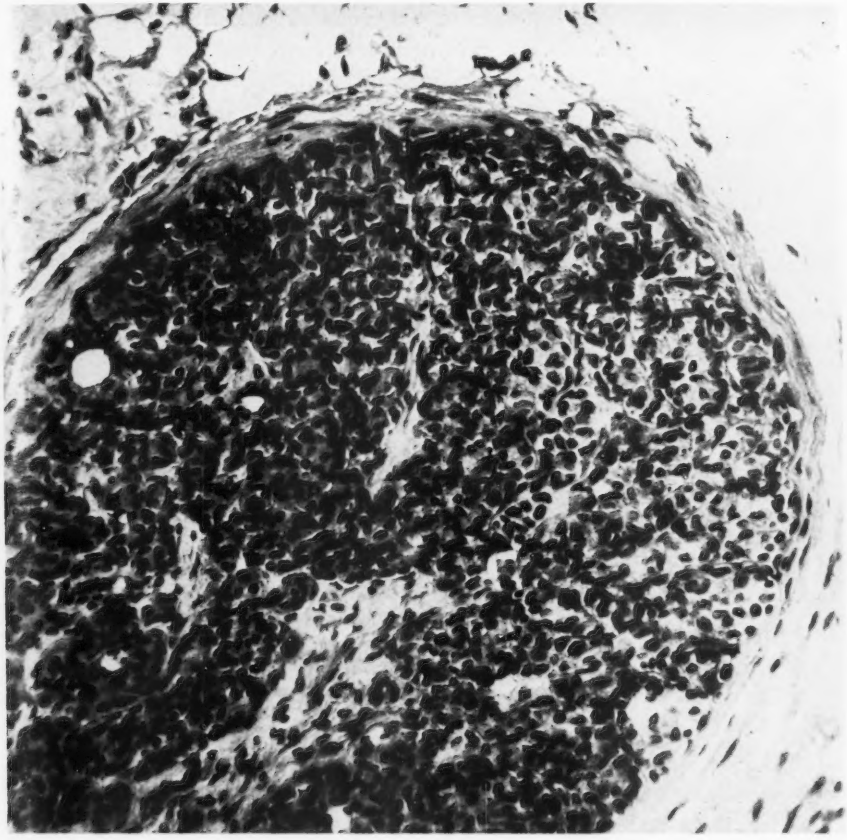

FIG. 4. Parathyroid from rat No. 2369; serum calcium 10.40 mg.% during normal delivery. Note conspicuous reticulum and variability of cellularity and grouping of cells —cords and sheets at the upper right and clusters at the lower left—reminiscent of normal parathyroid tissue. Sectioned at 6 μ, H and E $\times$220.

able parathyroid tissue was undoubtedly missed in many specimens. In spite of this technical error, it was still possible to make a rough correlation between the microscopic appearance of the parathyroid tissue and the condition of a particular animal at parturition. Even when the identification of each slide was concealed, it was possible to predict for 10 of 13 specimens from Experiment III that a "normal" or "very low" serum calcium level would be found. Parathyroid tissue was apparently missed during sectioning of one specimen from an animal with a serum calcium of 9.12 mg.%. In the second case, only one parathyroid was seen, and this was a remnant encapsulated with fiber, and chiefly composed of fibrous tissue with a very few cells; the serum calcium was low normal, 8.74 mg.%.

In the third case, again only one parathyroid was found. In this specimen one-third of the specimen was fibrous replacement tissue containing a few cells with darkly staining cytoplasm at the periphery; serum calcium was 10.49 mg.%.

A tetanic animal invariably had a serum calcium level of less than 6 mg.%. The histologic preparations from six animals in tetany usually indicated that the parathyroids had originally been deeply embedded in the thyroid tissue. The glandular remnants were surrounded by a thick fibrous capsule and contained small tightly clumped pyknotic or darkly staining cells in an extensive fibrous network.

There were four animals that were delivering normally, whose serum calcium was greater than 8 mg.%, and whose parathyroid histology fitted with their serum calcium level and general condition. Although fibrous invasion was prominent, the groups of cells retained their normal glandular structure; they had abundant cytoplasm, and the capillary bed was extensive. These four specimens had at least one parathyroid that appeared to have been on the surface of the thyroid gland before irradiation. This more favorable geometric location could account for the survival of much of the tissue which was only partially irradiated by the I^{131}. The presence or absence of structural damage could almost be judged on the basis of the spatial relationship of one or both parathyroids to the thyroid gland (embedded or on the surface)—at least insofar as this relationship could be reconstructed.

DISCUSSION

Our experiments support the view of many investigators that irradiation with I^{131} is a more effective means of thyroid destruction than surgical removal of the gland (43, 45, 46). However, this radiation procedure is not without side effects such as parathyroid damage and ovarian damage seen in I^{131} overdose (47, 48).

The 69% reduction in fertility in severely thyroid-deficient animals appears to be directly related to the absence of circulating thyroid hormone. Similar observations have been made by several workers (3, 4, 49, 50), although Hammet (50) did not consider his results conclusive.

The long physiological life of thyroid hormone—more than 40 days in the rat, according to Watts (13)—seems to be the source of many of the contradictions in studies of the relationships between thyroid deficiency and reproduction (10–12, 15). The long life of this hormone may help to explain, for example, the negative results of Bruce and Sloviter (15). These investigators used mice and bred them for the first time only 21 days after thyroid destruction with I^{131}. Some of these thyroidless mice conceived and bore as many as six litters. Females were mated continuously except during parturition and the 19 days while a litter was being suckled. This breeding schedule suggests at least one explanation for the fertility of their pres-

ably thyroid-deficient females. Taurog (51) found that L-thyroxine is normally excreted primarily in the feces of several species (rat, dog, sheep, rabbit). It passes from the bile through the gastrointestinal tract unchanged or as an enzymatically hydrolyzable conjugation product. Lactating rodents habitually consume the fecal matter of their nurslings; this would provide the thyroidless mothers with a ready source of L-thyroxine.

Fertility was nearly normal in our surgically thyroparathyroidectomized animals when thyroid supplements were given. Fertility was also nearly normal in rats whose thyroid tissue had been destroyed with I^{131} when triiodothyronine or thyroxine was given in amounts sufficient to maintain a normal standard metabolic rate. Judging from the number of pregnancies and the litter sizes of these animals, fertility did not seem to be impaired by the absence of the parathyroid glands, as suggested by Bodansky and Duff (19).

We were unable to demonstrate any beneficial effect of desiccated thyroid substance, thyroxine, or triiodothyronine on parturition. An increase in protein-bound iodine has been demonstrated in pregnant women near term (52). This substantial increase in thyroid hormone output at the end of gestation might have some effect on parturition, but no information on this point was available from these present experiments.

An acute need for serum calcium at term was not anticipated. Previous work gave no indication that severe parathyroid deficiency might be produced occasionally during radioiodine thyroid ablation. This lack of understanding of the difficulties encountered during parturition in the I^{131}-thyroidectomized rat was complicated by our own previous experimental work (44), as well as the observations of others (5, 43, 45, 46). Following the "discovery" of very low serum calcium levels (less than 6 mg.%), the parathyroid glands were specifically sought at necropsy, and histological preparations were made of peritracheal tissue.

The level of calcium in the Purina laboratory chow (1.35%) is apparently sufficient to maintain the parathyroid-deficient animals when not under stress. A large percentage of this calcium may be in an unutilizable form, since its source is fish meal and vegetable matter. This diet probably does not meet the standards for the high calcium diet recommended by Cox and Imboden (53) for support of parathyroidectomized rats. Augmenting this diet with additional calcium in the form of orally administered veterinary calcium gluconate appeared to have some beneficial effect (Experiment II) on the survival of the young. Calcium gluconate, U.S.P., used in Experiment III, did not aid maternal survival or improve litter survival (4 of 7 animals in the (T4 · Ca) group were in tetany at parturition). These observations suggest that oral administration of calcium in the form of pure calcium gluconate is of little use in ameliorating the symptoms of acute parathyroid deficiency.

An interesting observation was made in the two groups of surgically

thyroidectomized animals of Experiment I. Of a total of 46 pups that survived parturition, 24 died before they were more than 2 days old. The remaining 22 pups survived to weaning preceded by what appeared to be a 2-day period of malnutrition. This time period parallels some of the investigations of Munson (47), who found that parathyroidectomy in a lactating rat considerably reduced the calcium content of the milk for a period of 2 days. After this time the calcium concentration in the milk exceeded the concentration of calcium in the milk of normal lactating rats.

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The authors wish to thank Drs. Hardin B. Jones, I. L. Chaikoff, Edward S. Evans, Jr., and C. W. Asling of the Department of Anatomy and Physiology for their constant encouragement and helpful suggestions, and Mrs. Grace Walpole and Mrs. Helen Coble who prepared the manuscript.

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METABOLISM OF 3:5 DIIODO-L-THYRONINE BY L-AMINO ACID OXIDASE[1]

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ABSTRACT

3:5 Diiodo-L-thyronine (20–100 mg.) was incubated with crude L-amino acid oxidase of cobra venom (100–500 mg.) in (A) the absence or (B) the presence of additional catalase (30–120 mg.) for three hours at 37.3° C. (pH 7.4) and incubation mixture extracted with ether at pH 3.0. Chromatography of the ether extract from A in several solvent systems revealed in the main the presence of a phenolic compound, while that from B pointed to the presence of a carbonyl compound and a phenolic compound. The carbonyl compound was identical with an unknown degradation product of an authentic sample of 3:5 diiodothyropyruvic acid as established by chromatography or electrophoresis, color tests and ultraviolet absorbance. The phenolic compound had the same migration on chromatograms or electrophoregrams, and its crystals showed the same ultraviolet absorption and melting point as an authentic sample of 3:5 diiodothyroacetic acid. Under the same experimental conditions, N-chloroacetyl-3:5 diiodo-L-thyronine, in which the amino group of 3:5 diiodo-L-thyronine is inactivated with chloroacetylchloride, is not metabolized to the carbonyl and phenolic compounds.

On the basis of these results, it appears that during incubation with L-amino acid oxidase in the presence of large amounts of catalase 3:5 diiodothyronine is metabolized to 3:5 diiodothyropyruvic acid and that during chromatography and incubation this pyruvic acid derivative is decomposed to an unknown carbonyl compound and to 3:5 diiodothyroacetic acid.

IN PREVIOUS work from this laboratory it has been reported that I^{131}-labeled 3′:3:5 triiodo-L-thyronine and L-thyroxine are largely converted by crude L-amino acid oxidase of cobra venom to the corresponding acetic acid analogues and to small amounts of unknown compounds (1). Under the same conditions the major metabolites of I^{131}-labeled L-mono- and 3:5 diiodo-L-tyrosine were the corresponding pyruvic acid analogues and inorganic iodide (1, 2). On the other hand incubation of high concentrations of monoiodotyrosine with crude L-amino acid oxidase of cobra venom resulted in a greater conversion of the amino acid to the corresponding acetic acid analogue, suggesting a lack of sufficient catalase activity in cobra venom to decompose H_2O_2 during deamination (2). Roche and hi-

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co-workers (3) have suggested that triiodothyropyruvic acid and tetraiodothyropyruvic acid appear in rat bile and urine after the injection of pharmacological dosages of triiodothyronine and thyroxine. However, the identification of the above pyruvic acids in their work was limited to color tests without use of authentic samples.

The present work was undertaken to investigate the metabolic products which arise from 3:5 diiodo-L-thyronine under the effect of L-amino acid oxidase of cobra venom in the presence of crystalline catalase, added to lessen the nonenzymatic oxidation of the metabolite by H_2O_2 formed on autoxidation of the yellow enzyme.

MATERIALS AND METHODS

A. *Incubation Experiment.*

3:5 diiodo-L-thyronine[2] (20 mg.) or N-chloroacetyl-3:5 diiodo-L-thyronine (20 mg.) was dissolved completely in distilled water (20 ml.) by the addition of a minimum amount of 1N NaOH (approx. 10 drops) followed by 0.1 M phosphate buffer (pH 7.4) (200 ml.). The clear solution was mixed with a catalase[3] (30 mg.) and crude L-amino acid oxidase[4] (100 mg.) dissolved in 0.1 M phosphate buffer (20 ml.) and incubated for three hours at 37.3° C. in air in a two neck flask with an automatic stirrer. In several instances, the incubation with 3:5 diiodo-L-thyronine was carried out in the absence of additional catalase.

In all of the experiments with 3:5 diiodo-L-thyronine (T2), the incubation mixture became slightly turbid during incubation as a result of the precipitation of a small amount of starting material. After the mixture obtained in the experiments with T2 was brought to a pH of 3.0 with concentrated hydrochloric acid, it was extracted once with 2 volumes of ether redistilled from $FeSO_4$ and twice with equal volumes of the ether. Most of the metabolites of T2 were extracted by this procedure. The residual aqueous solution was then extracted three times with equal volumes of n-butylalcohol. In the experiments with N-chloroacetyl 3:5 diiodo-L-thyronine (N-CA-T2), the incubation mixture was extracted either three times with ether by the same procedure as in the T2 experiments or four times with equal volumes of ethylacetate at a pH of 7.4. In both sets of experiments the pooled ether extracts were evaporated to dryness *in vacuo* in an atmosphere of nitrogen. To reduce the salt content the n-butylalcohol extract was taken up in 20 ml. of mixture of n-butylalcohol and concentrated ammonia (50:1), washed once with one-half volume of water and then evaporated to dryness *in vacuo* in an atmosphere of nitrogin.

B. *Chromatographic and Electrophoretic Separation of Products.*

The above residues from each set of experiments were dissolved in small amounts of ethanol or n-butylalcohol saturated with concentrated ammonia, a sample was placed

[2] 3:5 Diiodo-L-thyronine was kindly supplied by Dr. R. I. Meltzer (Warner-Lambert Research Institute, Morris Plains, New Jersey) and by Dr. R. S. Johnson (Smith Kline and French Laboratories, Philadelphia, Pa.)

[3] Crystalline catalase was obtained from General Biochemicals Inc., Laboratory Park, Chagrin Falls, Ohio.

[4] Crude cobra venom was obtained from Hynson, Westcott and Dunning, Inc., Baltimore and used as source of L-amino acid oxidase without purification.

on Whatman No. 1 filter paper and developed chromatographically by the descending method.

The following solvents were employed in the chromatography system: 1) tertiary amylalcohol saturated with 2N ammonia; 2) n-butylalcohol-2N ammonia-dioxane (4:2:1); 3) collidine-water (100:35.5) in ammonia atmosphere; and 4) n-butylalcohol-acetic acid-water (100:15:37). The electrophoregrams of the products were obtained by means of Model E 800-2 (Research Specialties Co.) in an electric field of about 8 volt/cm. for 5 hours. A buffer of 0.05 M. $(NH_4)_2 CO_3$ and Whatman No. 1 filter paper were used. The following color reagents were employed for the location of compounds on the paper chromatograms and electrophoregrams: 1) diazotized sulfanilic acid and 5% Na_2CO_3 in 50% ethanol (4); 2) 2:4 dinitrophenylhydrazine saturated with 1N HCl (when the reagent was sprayed lightly the carbonyl compound spots assumed a deep yellow color) (1); 3) o-phenylendiamine (5); 4) semicarbazide (6); 5) alpha-nitroso-beta-naphthol (7); 6) 0.1% ninhydrin in n-butylalcohol saturated with water (after spraying the paper was heated to 80–100° C.); and 7) 1% $PdCl_2$ in water (4).

C. *Synthesis of Iodinated Compounds.*

1. *N-chloroacetyl-3:5 diiodo-L-thyronine (N-CA-T2).* This compound has been made by Ashley and Harington by indirect acylation of T2 (8) but the polarity was not reported.

a) *Indirect method.* The compound was prepared by a modification of the method described by Ashley and Harington (8), using a mixture of acetone and anisole (2:1) instead of anisole alone as the solvent for acylation of 3:5 diiodo-L-thyronine methylester (second stage product). Important products formed during the four stage process of this synthesis included: 1) N-chloroacetyl-3:5 diiodo-L-thyronine-methylester: long needles, m.p. 172° C., $(\alpha)^{26.5}D = +6.91°$ (9.225 mg. in 1 ml. of ethanol), (Analysis Calculated for $C_{18}H_{16}O_5NCl\ I_2$: C, 35.1; H, 2.6; N, 2.3; I, 42.1. Found: C, 35.4; H, 2.7; N, 2.1; I, 42.6%); 2) N-chloroacetyl-3:5 diiodo-L-thyronine: fine needles, m.p. 146° C. after sintering at 134° C, $(\alpha)^{25.5}D = +33.51°$ (6.790 mg. in 1 ml. of ethanol), (Analysis Calculated for $C_{17}H_{14}O_5NCl\ I_2$: C, 33.9; H, 2.3; N, 2.4; I, 42.2. Found: C, 33.5; H, 2.7; N, 2.4; I, 41.7%).

b) *Direct method.* T2(1.2 gm.) was suspended in a mixture of ethylacetate (120 ml.) and chloroacetylchloride (1.2 ml.). The mixture was then refluxed for 20 minutes, and the slightly turbid solution evaporated to dryness *in vacuo*. The residue taken up in ethylacetate (25 ml.) was filtered to remove insoluble material; the filtrate was then concentrated to about 3 ml. *in vacuo* at 50° C. followed by the addition of benzene (20 ml.). After overnight storage at 4° C, the crude crystals were collected and washed with benzene: yield 1.12 gm. (81%). Crystallization from 30% ethanol gave colorless fine needles, m.p. 146° C. after sintering at 134° C. which showed no melting point depression with N-CA-T2 obtained by the indirect method. $(\alpha)^{25.0}D = +35.5°$ (6.58 mg. in 1 ml. of ethanol). Analysis calculated for $C_{17}H_{14}O_5NCl\ I_2$: N, 2.4; I, 42.2. Found: N, 2.0; I, 39.6%.

2. *α-Acetylamino-4-p-hydroxyphenoxy-3:5 diiodo-cinnamic acid (AC-T2C).* This compound was prepared by a two stage process from N-CA-T2: 1) Azlactone of α-acetylamino-β (4-p-acetoxyphenoxy-3:5 diiodophenyl) acrylic acid—N-CA-T2 (0.916 gm.) was dissolved in dry pyridine and treated with acetic anhydride (9.2 ml.) for 30 minutes at room temperature. After cooling for 2 hours, the resultant crystals were collected and washed with absolute ethanol: yield 0.48 mg. m.p. 232–234° C. Also a crude azlactone was obtained by pouring the mother liquor into ice-water: the resulting brown precipitate washed with alcohol and ether weighed 0.31 gm. (m.p. 222–230° C.). The total yield was 88.1% of theoretical. Crystallization from hot glacial acetic acid resulted in slightly yellowish needles which melted at 230–236° C. (dec.). Analysis calculated f

$C_{19}H_{13}O_5N\ I_2$: C, 38.7; H, 2.2. Found: C, 39.0; H, 2.4%. 2) AC-T2C—The above azlactone (0.1 gm.) was boiled in 1N NaOH (3 ml.) for about 10 minutes. The slightly turbid yellowish solution was then filtered and the mother liquor acidified with 2N HCl. After cooling for several hours, the crystals were collected and washed with water. Dilution of an alcohol solution of the crystals with water yielded slightly yellowish, fine needles with a m.p. 210–212° C. (dec.), yield: 0.059 gm. (62.2%). Analysis calculated for $C_{17}H_{13}O_5N\ I_2$: C, 36.1; H, 2.3; N, 2.5; I, 44.9. Found: C, 35.6; H, 2.7; N, 2.3; I, 43.8%.

3. *3:5 Diiodothyropyruvic acid*[5] (*T2K*). The above azlactone (0.925 gm.) was refluxed in 30% KOH (9 ml.) for one hour. The reddish yellow solution was then allowed to stand overnight at 4° C. and the resulting fine crystals were collected. The filtrate and the crystals in water were saturated with sulfur dioxide and the precipitates obtained from both solutions were combined and thoroughly washed with ether. The product was then dissolved in hot water by the addition of small amounts of sodium carbonate and brought to a pH of 4.0 with 1N HCl at the boiling point. This solution was extracted four times with distilled ether. The pooled ether extracts were dried over sodium sulfate and evaporated to dryness; the resulting residue was crystallized from 30% acetic acid with the appearance of colorless fine prisms (m.p. 185–189° C.). The yield was 0.246 gm. (30%). Analysis calculated for $C_{15}H_{10}O_5\ I_2$: C, 34.4; H, 2.1; I, 48.4. Found: C, 34.5; H, 2.2; I, 47.8%.

4. *3:5 Diiodo-methoxy-thyropyruvic acid* (*MO-T2K*). This compound was prepared by the method of Canzanelli *et al.* (9).

RESULTS

I. Experiments with T2

(In the Presence of Additional Catalase)

A. *Metabolites in the Ether-Extractable Fraction of the Incubated Mixture.*

As shown in Figure 1 two spots were found on paper chromatograms in systems 1, 2, and 3 by spraying a diazotized sulfanilic acid reagent: a purple spot (T2U1) and an orange spot (T2U2). In all solvent systems, T2U1 moved more slowly than T2U2. One spot, brown-red in color after spraying with the diazotized sulfanilic acid reagent, was observed after chromatography in system 4. From several color tests (Table 1) it appears likely that T2U1 is a phenolic compound without a carbonyl group or amino group and that T2U2 is a phenolic compound with a carbonyl group. Since T2U1 and T2U2 gave a positive α-nitro-β-naphthol test, iodine replacement in the 3′ and 5′ position should not occur in these molecules. No inorganic iodide was detected on the chromatograms by $PdCl_2$.

1. *Identification of T2U1.* When the ether extract was mixed with an authentic sample of 3:5 diiodothyroacetic acid[6] (T2A) and run in system 1, 2 or 3, the migration of T2U1 was identical with that of T2A. To identify T2U1 further, the experiment with 20 mg. of T2 was repeated four times. The ether extract from each incubation mixture was combined and evapo-

[5] We have since learned that Drs. H. T. Kennedy and R. Pitt-Rivers have also prepared this compound by a similar method (communication from Dr. R. Pitt-Rivers).

[6] Pure 3:5 diiodothyroacetic acid (m.p. 215–216° C.) was kindly supplied by Dr. J. S. Johnson.

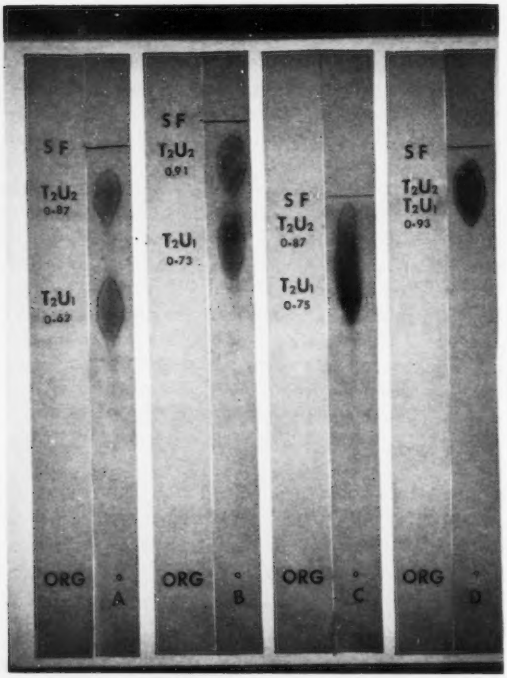


FIG. 1. Paper chromatography of the ether-extractable fraction of the incubation mixture of T2 with crude L-amino acid oxidase and catalase.

The residue from the ether-extractable fraction in 0.5 ml. absolute ethanol (8 μl.) was applied on paper and developed in: A) system 1 for 24 hours; B) system 2 for 13 hours; C) system 3 for 16 hours; and D) system 4 for 12 hours. The position of compounds on the chromatograms was located by color development with diazotized sulfanilic acid.

ORG = origin; SF = solvent front; numbers under the letters refer to the Rf values of T2U1 and T2U2.

TABLE I

Groups	Color reagents	T2U1	T2U2
Phenolic	Diazotized sulfanilic acid	Purple (+)	Orange (+)
	α-Nitro-β-naphthol	Light orange (+)	Light Orange (+)
Carbonyl	2:4 Dinitrophenyl hydrazine	(−)	Yellow (+)
	O-phenylendiamine*	Black shadow (−)	Deep black shadow (+) (colored slightly yello green)
	Semicarbazide*	Black shadow (−)?	Deep black shadow (+)
Amino	Ninhydrin	(−)	(−)

* Both T2U1 and T2U2 revealed black shadows under the ultraviolet lamp without spra ing O-phenylendiamine or semicarbazide reagent.

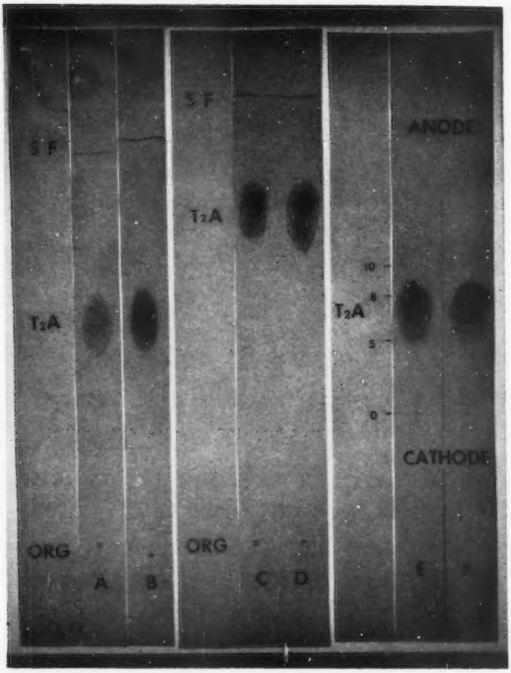


FIG. 2. Re-chromatography of T2U1, co-electrophoresis and co-chromatography of T2U1 with T2A.

Re-chromatography of T2U1 (50 μg.) in: A) system 1; C) system 2. Co-chromatography of T2U1 (50 μg.) with T2A (50 μg.) in: B) system 1; D) system 2. Electrophoresis of T2U1 (50 μg.) and co-electrophoresis of T2U1 (25 μg.) with T2A (25 μg.) in 0.05 M $(NH)_2CO_3$ buffer in E and F, respectively. Symbols as in Figure 1. Numbers written on the left of the electrophoregrams refer to the distance between the origin and the anode (cm.); color reagent: diazotized sulfanilic acid.

rated to dryness on a water bath. The resulting oily residue was then dissolved in absolute alcohol (about 0.8 ml.), applied lineally in 40 cm. lengths on each of four sheets of Whatman No. 1 filter paper, and chromatographed in system 1 for 24 hours at room temperature. After drying for 6 hours in a hood two bands (T2U1 and T2U2), appearing as visible dark shadows under an ultraviolet lamp,[7] were cut from each paper. The paper strips containing T2U1 or T2U2 were eluted with 80% ethanol overnight at room temperature and evaporated to dryness *in vacuo* in an atmosphere of nitrogen. The residue obtained from the paper containing T2U1 was then extracted with 2 ml. of absolute alcohol and diluted with about 5 volumes

[7] To minimize decomposition of compounds in the chromatogram exposure under the ultraviolet lamp was limited to less than 15 seconds.

of water. One drop of glacial acetic acid was added at this point. After overnight storage at 6° C. the crystalline precipitate was collected by centrifuging, washed with water, and dried over P_2O_5 *in vacuo* at 78.5° C.; m.p. 206–208° C., yield 27.6 mg. From electrophoresis and chromatography (Fig. 2) it was evident that this material contained little if any phenolic compounds and had the same migration as an authentic sample of T2A. This material dissolved in 60% ethanol gave negative $FeCl_3$ test (9) and

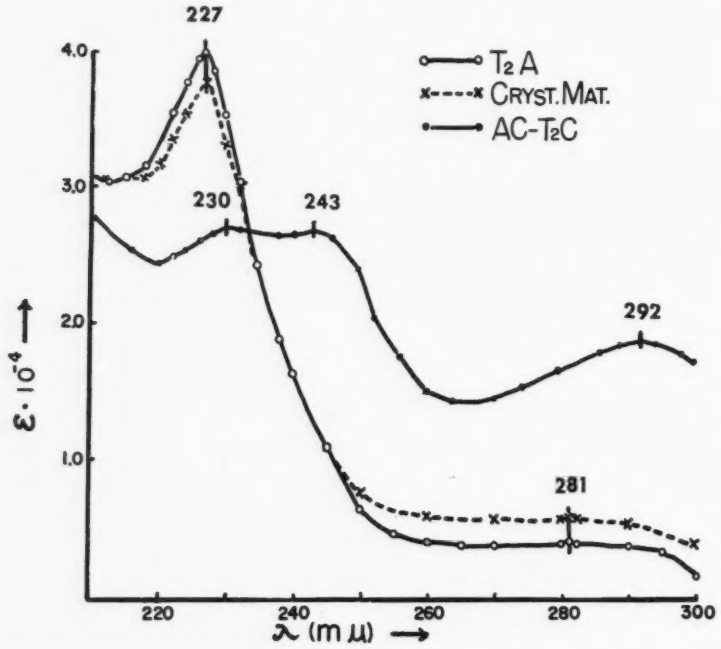


FIG. 3. Ultraviolet spectra of T2A, AC-T2C and a crystalline substance isolated from T2U1.

The compounds were dissolved in absolute ethanol. The crystalline substance was weighed as T2A.

2:4 dinitrophenylhydrazine tests (10). Dilution of this powder (20 mg.) in absolute alcohol (1.5 ml.) with four volumes of water produced slightly yellowish rhombic plates: m.p. 212.5–213° C., yield 16.8 mg. The mixed melting point with an authentic sample of T2A was not depressed. As indicated in Figure 3, the crystalline material exhibited peak absorbances at 227 mμ and 281 mμ identical with that of an authentic sample of T2A. Its absorption curve was therefore strikingly different from that of AC-T2C. These physico-chemical and chemical analyses show clearly that most of T2U1 consists of T2A.

2. *Identification of T2U2.* When the ether-extractable fraction of the incubation mixture was mixed with an authentic sample of T2K and run in system 1, 2 or 3, only one visible spot (T2U2) was found on the chromatograms after spraying with 2:4 dinitrophenylhydrazine reagent. The evaporation of the elutant from paper which contained T2U2 (described above) resulted in a yellowish oily residue which solidified following the addition of 30% acetic acid. This solid mass washed with water and dried

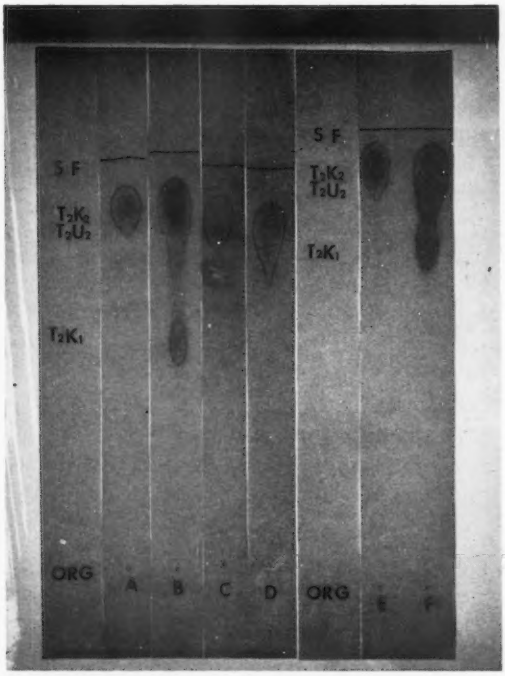


FIG. 4. Re-chromatography and co-chromatography of T2U2 with T2K.

Re-chromatography of T2U2 (50 μg.) in system 1 (A, C) and in system 2 (E), chromatography of T2K (80 μg.) in system 1 (D). Co-chromatography of T2U2 (50 μg.) with T2K (50 μg.): B) in system 1; F) in system 2. Color reagent: diazotized sulfanilic acid for A, B, E, F; 2:4 dinitrophenylhydrazine for C and D. Symbols as in Figure 1.

over P_2O_5 *in vacuo* at 78.5° C. yielded 20.8 mg. of a sticky yellowish material. When this was rechromatographed in several solvent systems, only one spot was found after spraying with either diazotized sulfanilic acid (Figs. 4 A and E), 2:4 dinitrophenylhydrazine (Fig. 4 C) or α-nitroso-β-naphthol. This material did not move from the origin to the cathode or the anode for 5 hours under the electrophoretic conditions mentioned in the method. On the other hand, chromatography of the mixture of the authentic samples of T2K and T2U2 in system 1 (Fig. 4 B) or in system 2

(Fig. 4 F) revealed two spots after spraying with diazotized sulfanilic acid. One visible spot which showed a positive 2:4 dinitrophenylhydrazine test had the same migration as T2U2 and the other which was slightly positive in the 2:4 dinitrophenylhydrazine test had the same migration as T2A. Two spots showing color tests similar to those of T2U2 and T2A were also obtained when an authentic sample of T2K was chromatographed in system 1 (Fig. 4 D) or in system 2. These were named T2K2 and T2K1 respectively. The ultraviolet absorption of these compounds isolated from T2K (40 mg.) by paper chromatography in system 1 was determined after elution and evaporation in accord with the procedure employed with T2U1 and T2U2. Following the dilution of T2K1 in absolute alcohol with water, a crystalline powder (3.9 mg.) showing a m.p. of 210° C and maximal absorbances of 227 and 281 mμ was obtained, and concentration of the mother liquor gave rise to a light precipitate showing positive $FeCl_3$ (9) and 2:4 dinitrophenylhydrazine tests (10). An attempt to isolate T2K as crystals was unsuccessful; T2K1 would appear to be an admixture of T2A and T2K. On the other hand, T2K2 (5.5 mg.) was isolated as a sticky yellow powder. Its chemical properties were strikingly different from those of T2K and MO-T2K, but identical with those of T2U2 (Fig. 5 and Table 2). In view of these results, it seems likely that an authentic sample of T2K is partly decomposed to T2A and the unknown carbonyl compound during chromatography in system 1 or 2. Therefore, a part of T2A and the unknown compound isolated from the ether-extractable fraction of the incubation mixture by paper chromatography in system 1 are probably degradation products of T2K which appear during the chromatographic procedure, if T2K has been formed from T2 during incubation.

B. *Compounds in the n-Butylalcohol Extract of the Water Layer Obtained After Extraction of the Incubation Mixture with Ether.*

As shown in Figure 6, two spots giving positive ninhydrin and diazotized sulfanilic acid tests were found after chromatography in system 1. These were identical with those obtained on chromatography when T2 was used as the starting material in the incubation experiment. Of the two, one (T2U0) yielded only a very faint color with the above reagents. It is unlikely therefore that T2U0 is a metabolite of T2 produced by crude L-amino acid oxidase with added massive dosages of catalase. No inorganic iodide was found on the chromatograms after spraying by $PdCl_2$.

II. Experiments with T2

(Without Added Catalase)

When the ether extractable fraction from this incubation medium was evaporated to dryness, fine colorless crystals appeared. Chromatograph-

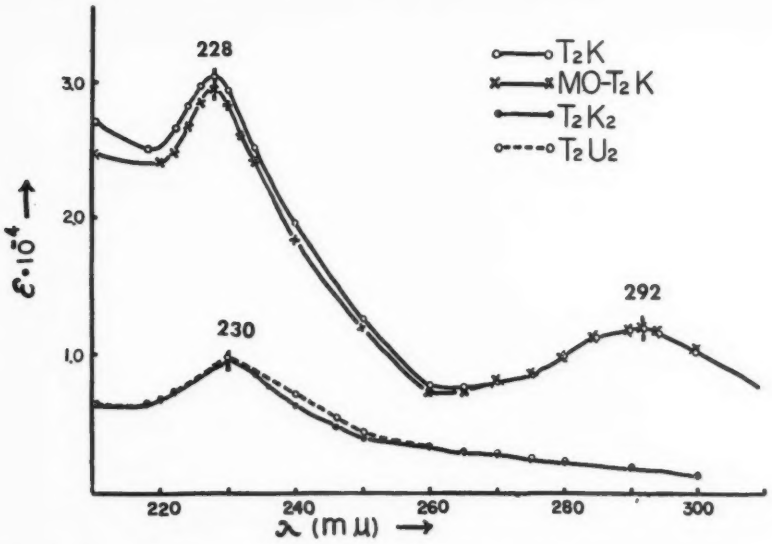


FIG. 5. Ultraviolet spectra of T2U2, T2K2, T2K and MO-T2K.

The authentic T2K and MO-T2K were dissolved in absolute ethanol to yield a 10^{-5} M. solution. T2U2 and T2K2 were weighed as T2K, and dissolved in absolute alcohol as described above.

of the crystalline residue in 0.5 ml. absolute alcohol (8 μl) (in system 1, 2, or 3) produced an intense color spot which showed the same migration and color tests as an authentic sample of T2A, and a very faint spot with the same migration and color reactions as T2U2 or T2K2. One dilution of the alcoholic solution with an equal volume of water promptly produced fine and colorless rhombic crystals which melted at 214–215° C. Admixture with an authentic sample of T2A did not depress the melting point. On the other hand, chromatography of the n-butylalcohol extractable fraction of the water layer obtained after extraction of the incubation mixture with ether in system 1 revealed two spots which gave a positive ninhydrin test,

TABLE II

	T2K		MO-T2K		T2U2		T2K2	
$FeCl_3$ *	Green (+)		Green (+)		(−)		(−)	
2:4 Dinitrophenyl-** hydrazine and NaOH	(+)		(+)		(+)		(+)	
	Max. peak: 444 mμ				Max. peak: 433 mμ		Max. peak: 433 mμ	
Ultraviolet spectra (in alcohol)	Peaks(mμ)	$\epsilon \times 10^{-4}$	Peaks(mμ)	$\epsilon \times 10^{-4}$	Peak(mμ)	$\epsilon \times 10^{-4}$	Peak(mμ)	$\epsilon \times 10^{-4}$
	228 292	3.05 1.2	228 292	2.95 1.2	230	0.96	230	0.95

$FeCl_3$ in absolute ethanol described by Canzanelli *et al.* (9): this reagent reacts with all pyruvic acid analogues of tyrosine, monoiodotyrosine, diiodotyrosine and thyroxine in absolute alcohol.

* This test was carried out by the modification of the method described by L. Penrose and J. H. Quastel (10), using 50% ethanol instead of water as the solvent of the carbonyl compounds.

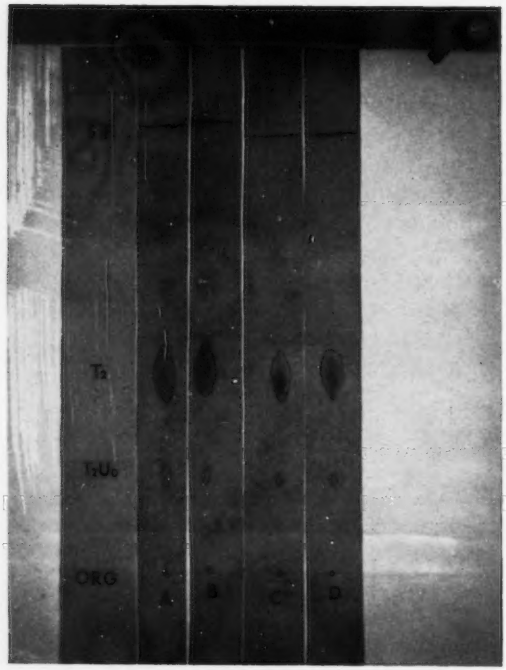


FIG. 6. Paper chromatography of T2 and the n-butylalcohol-extractable fraction of the water layer obtained after extraction of the incubation media with ether.

A and C: 8 μl. of the residue from the n-butylalcohol extractable fraction in 0.20 ml. n-BuOH alkalized with ammonia. B and C: 50 μg. of T2 in n-BuOH alkalized with ammonia. Solvent: System 1. Color reagent: diazotized sulfanilic acid for A and B, ninhydrin for C and D.

identical with that seen in the incubation experiments carried out in the presence of additional catalase. Chromatography and spraying with $PdCl_2$ did not reveal any inorganic iodide either in the ether extract or the butanol extract.

III. Experiments with N-CA-T2

When the ether-extractable fraction of the incubation mixture of N-CA-T2 with crude L-amino acid oxidase and catalase was chromatographed in system 1, one spot giving positive diazotized sulfanilic acid and α-nitroso-β-naphthol but negative ninhydrin tests was found on the papers. As shown in Figure 7, this spot was located under the position assumed by an authentic sample of T2A (Figs. 7A, B, and C). It showed the same Rf as an authentic sample of N-CA-T2 when the Rf value was calculated from the origin to the top of the spot (Figures 7 A and B). Chromatography of the ethylacetate-extractable fraction of the incubation

mixture in system 1 revealed an elongated spot which reacted with diazotized sulfanilic acid reagent (Figs. 7 E, F and G), identical with that seen with an authentic sample of N-CA-T2 (Fig. 7 D). On the other hand, a round spot which had the same Rf as that seen with the ether-extractable fraction was obtained on chromatograms in system 2 (Figs. 7 H and I). To identify the compound further the incubation mixture of N-CA-T2 (20 mg.) with crude L-amino acid oxidase and catalase was extracted four times with equal volumes of ethylacetate at a pH of 7.4. The pooled extract was evaporated to dryness *in vacuo* as described above. Dilution of the residue in absolute alcohol with four volumes of water yielded a crystalline precipitate. After cooling overnight, the precipitate was collected, washed with water, and dried over P_2O_5 *in vacuo:* Yield 13.9 mg. Small

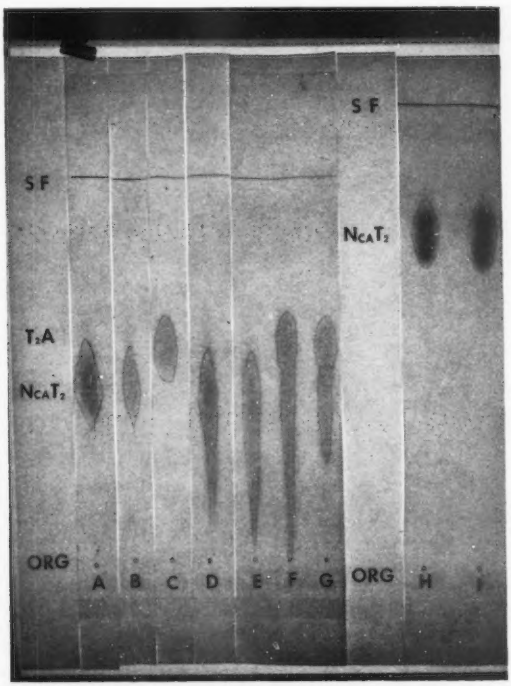


FIG. 7. Paper chromatography of ether-extractable and ethylacetate-extractable fraction of the incubation mixture of N-CA-T2 with L-amino acid oxidase, authentic N-CA-T2, and authentic T2A.

A: residue from the ether extract in 0.5 ml. ethanol (8 μl.). B: same alcoholic solution as A (4 μl.). C: authentic T2A (80 μg.). D: authentic N-CA-T2 (80 μg.). E: residue from the ethylacetate extract in 0.5 ml. ethanol (8 μl.). F: mixture of T2A (80 μg.) and N-CA-T2 (80 μg.). G: mixture of T2A (80 μg.) residue from the ethylacetate in 0.5 ml. ethanol (4 μl.). H: residue from the ether extract in 0.5 ml. ethanol (8 μl.). I: residue from the ethylacetate in 0.5 ml. ethanol (8 μl.). Color reagent: diazotized sulfanilic acid.

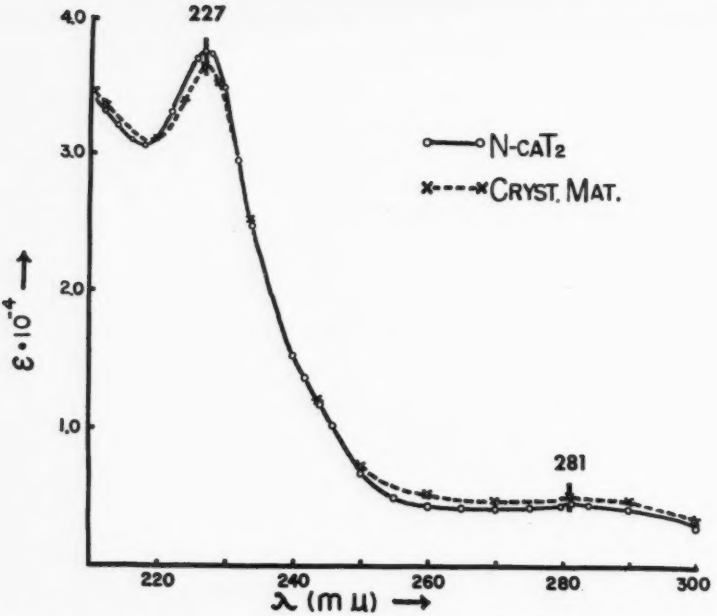


FIG. 8. Ultraviolet spectra of N-CA-T2, and a crystalline material isolated from the incubation mixture of N-CA-T2 with crude L-amino acid oxidase in absolute ethanol.
The crystalline material was weighed as N-CA-T2 and dissolved in absolute ethanol.

needle crystals of this material melted at 146° after sintering at 134° C. The mixed melting point with an authentic sample of N-CA-T2 was not depressed. In addition, the ultraviolet absorption curve of this crystalline material coincided almost exactly with that of an authentic sample of N-CA-T2 (Fig. 8).

From these results, it is clear that N-CA-T2 is not converted to T2A, T2U2 nor any other phenolic compounds, and that more than 70% of the starting compound is recovered by extraction of the incubation mixture with ethylacetate.

DISCUSSION

Since the radioactive iodothyronines (thyroxine and triiodothyronine) which have been generally used in *in vitro* or *in vivo* experiments have been labeled in the 3′ or 3′ and 5′ position, radioautography of paper chromatograms would not have detected deiodination to T2 in animal tissues. Radioactive T2 is formed however in the thyroid of the rat after injection of I^{131} (11) and consequently would be present in peripheral tissues after proteolysis of the thyroglobulin. In the experiments described in this paper T2 was readily metabolized to ether-extractable substances by crude L-amino acid oxidase in the presence or absence of additional catalase during a three-hour incubation period. Chromatography of the ether-extractable

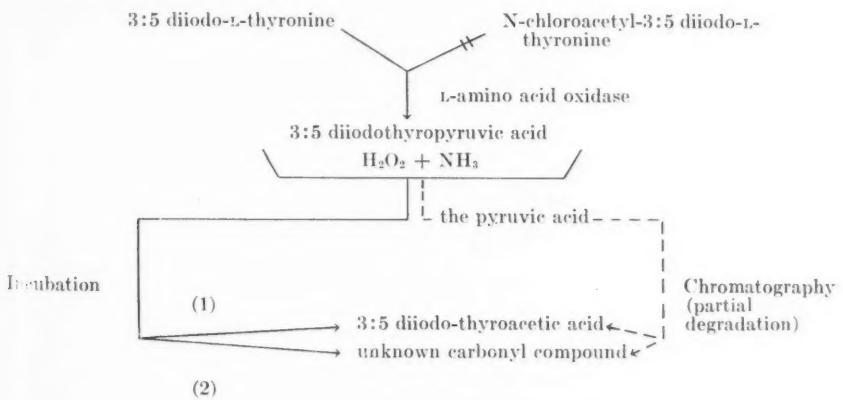
fraction from the experiments with additional catalase (in alkaline solvent systems) revealed the presence of carbonyl and phenolic compounds. After isolation of these two compounds from paper chromatograms and crystallization, one of them proved to be T2A which separated out as rhombic crystals. The other was a carbonyl compound in the form of a yellow powder believed to be a degradation product of T2K. On the other hand, an ether extract obtained from the incubation experiments carried out in the absence of added catalase contained large amounts of T2A which separated out without the aid of chromatography. This suggested the presence of T2A in high concentration in the ether extract. This was established to be indeed the fact by chromatography of the ether extract.

Since chromatography of analytically pure T2K in alkaline solvent systems revealed the presence of the above two compounds (T2A and the carbonyl compound) and also T2K unchanged, it appears likely that T2 is converted to T2K by L-amino acid oxidase during incubation and the resulting T2K is partially decomposed to T2A and the unknown carbonyl compound during chromatography. T2K synthesized by our method is believed to have been an essentially pure compound, because its chemical analysis was identical with its calculated composition and its ultraviolet absorbance was quite similar to that of methoxy-3:5 diiodothyropyruvic acid in absolute alcohol.

It is of interest that N-chloroacetyl-3:5 diiodo-L-thyronine in which the amino group is protected by acylation was not metabolized to carbonyl and phenolic compounds by crude L-amino acid oxidase and catalase.

It can be concluded therefore that the deamination of T2 occurs under the influence of the L-amino acid oxidase but not the other enzymes present in cobra venom, under the conditions used in these experiments.

It would appear that T2 is deaminated by L-amino acid oxidase to T2K and decomposed during chromatography in alkaline solvent systems and by H_2O_2 formed on autoxidation of L-amino acid oxidase during incubation as shown in the following scheme:



If concentration of the substrate (3:5 diiodothyronine) and the activity of L-amino acid oxidase are constant, the main metabolic pathway of the pyruvic acid will be (1) in the absence of catalase activity, and both (1) and (2) during an increase in catalase activity. Probably the unknown carbonyl compound is formed from the pyruvic acid as precursor of the acetic acid and oxidized to the acetic acid. Recently, a carbonyl compound showing the same color reaction as the unknown carbonyl compound, which seems to be 3:5 diiodo-thyroaceto-aldehyde, has been obtained as a crude powder by the oxidation (deamination and decarboxylation) of 3:5 diiodo-L-thyronine with ninhydrin in butanol-water (9:1) (12). On the basis of paper chromatographic findings it has been reported that radioactive thyroxine and 3′:3:5 triiodothyronine are converted in the main to their corresponding acetic acid analogues by crude L-amino acid oxidase of cobra venom which contains some catalase, even though catalase is not added (1). However the two acetic acid analogues could also be degradation products of their corresponding pyruvic acid analogues appearing during chromatography and incubation, comparable to the T2A formed in the present experiments with T2. Therefore further studies of the metabolism of triiodothyronine and thyroxine in the presence of pure L-amino acid oxidase of cobra venom with or without added catalase are indicated. It might be however that use of pure L-amino acid oxidase of cobra venom which has been freed of catalase by itself results in a complete conversion of pyruvic acid analogues of iodothyronines to their corresponding actic acid analogues without any formation of carbonyl compounds. Even though Tomita (13) has reported on the basis of findings with paper chromatography that diiodothyropyruvic acid labeled with C^{14} in the carbonyl position is rapidly converted by an extract of rat kidney mitochondria to radioactive diiodothyroacetic acid, the nature of this starting material and its metabolites were not described in his report.

In 1955, Taborsky (13) proposed that an α,β-unsaturated tyrosine was formed during the oxidative deamination of L-tyrosine by ophio-L-amino acid oxidase. He based this suggestion on the results of measurements of the ultraviolet spectra of incubation media. Since the characteristic ultraviolet spectrum of the acylated-α,β-unsaturated 3:5 diiodothyronine (AC-T2C) is due to the two conjugated double bands in its molecule (having no relation to the presence or absence of acetylchloride group in NH_2 group), α,β-unsaturated diiodothyronine or its related compounds would exhibit a similar curve to AC-T2C in ultraviolet region. However, neither of the two compounds isolated chromatographically from the ether-extractable fraction of the incubation mixture with T2 showed an ultraviolet absorbence identical with that of an authentic sample of AC-T2C. Also, neither of the two compounds separated chromatographically from the n-butylalcohol extract (ether-unextractable fraction) could be unsaturated diiodothyronine, even though they gave a ninhydrin test. Even if the α,β-

unsaturated amino acid were formed from T2 during incubation experiments with crude L-amino acid oxidase, it would not be detected by paper chromatography because of its supposed instability.

Generally, amino acids are deaminated to their corresponding pyruvic acid analogues by removal of the amino group by transaminases or through oxidation by L-amino acid oxidase. A part of the deamination of T2 in animal tissues may be effected by L-amino acid oxidase, just as it occurs *in vitro* with the crude L-amino acid oxidase of cobra venom.

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ISOLATION AND IDENTIFICATION OF ESTRIOL GLUCOSIDURONATE IN HUMAN MECONIUM[1]

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In Memoriam of Dr. W. S. Bauld

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ABSTRACT

A method is described for the extraction and purification of estriol glucosiduronate from various sources. By the use of this method estriol glucosiduronate has been isolated and identified in extracts of human meconium. Another conjugated form, which might be an estriol sulphate has also been detected.

IT HAS been reported previously, that human meconium has a rather peculiar estrogen pattern, since it contains a very high concentration of estriol (1, 2), but very little, if any estrone, 17β-estradiol, 16-*epi*estriol, 16α-hydroxyestrone, 16β-hydroxyestrone, or 16-oxo-17β-estradiol (2). Approximately 85% of the estriol in meconium was found in a "conjugated" form (2). Since recent evidence obtained in this laboratory indicates that the human fetus itself is capable of conjugating estriol *in utero* (3), it appeared to us of interest to study the nature of the conjugated estriol present in the meconium.

In this paper we wish to report on the isolation and identification of estriol glucosiduronate in extracts of meconium. Another conjugated form, which occurs in approximately equal amounts has also been isolated, but not yet identified.

EXPERIMENTAL

Collection of meconium.—Meconium was collected from apparently healthy male infants, using the method described previously (2). Meconium collected on the 2nd to 4th day of life was pooled and kept in a refrigerator at −5° C until processed. Extraction was carried out within a week following the completion of the collection.

Reagents.—Analytical grade reagents were used. The organic solvents employed were redistilled through fractionating columns.

Paper chromatography.—Descending chromatograms of the Bush-type (4) were run at 21° C on Whatman No. 2 paper. Equilibration time was 16 to 24 hours and running time 4 to 20 hours, depending on the solvent system used.

Paper electrophoresis.—An LKB 3276 BN paper electrophoresis apparatus (LKB-Produkter, Stockholm) and Whatman No. 2 paper were used. The potential was 300

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volts and the current 8 to 10 mA. The running time was 16 hours. Buffer solutions:

a) Phosphate buffer: 0.05M monopotassium phosphate and 0.05M disodium phosphate; pH 6.8, with 20% absolute ethanol added.

b) Veronal buffer: 0.05M sodium diethylbarbiturate, 0.005M diethyl barbituric acid and 0.05M sodium acetate; pH 8.6, with 20% absolute ethanol added.

Development of spots.—Following paper chromatography or paper electrophoresis, the papers were allowed to dry at room temperature and were developed either with the Folin-Ciocalteau reagent (5), or according to Barton *et al.* (6).

Countercurrent distribution.—All distributions were carried out in a 24-transfer all glass apparatus, manufactured according to the specifications of Craig and Post (7). At the end of the distribution the contents of each tube were evaporated to dryness and analyzed for estriol. Theoretical distribution values were calculated according to the method of Way and Bennett (8). In some cases (for instance in systems containing quinoline) only one phase was analyzed; methods for the calculation of theoretical distribution values in such cases are discussed *i.a.* by Hecker (9). Some of the solvent systems developed for the purposes of the present investigation are shown in Table 4.

Enzymic hydrolysis.—Two enzymes were used: one prepared from the digestive tract of Helix pomatia, containing both β-glucuronidase and sulphatase activities (L'industrie biologique française, Gennevilliers, France) and a bacterial β-glucuronidase preparation (Sigma Chemical Co., Saint Louis, Mo., U.S.A.). With the Helix pomatia enzyme hydrolysis was carried out in 0.1M (secondary sodium citrate) buffer at pH 5.0 and 37° C for 24 hours. The final concentration of the enzyme in the incubation mixture was 2000 Fishman units of β-glucuronidase and 1000 sulphatase units per ml. With the bacterial β-glucuronidase hydrolysis was carried out in 0.1M. (phosphate) buffer at pH 6.8 and 37° for 48 hours. Final enzyme concentration was 500 Fishman units per ml. The β-glucuronidase activity of the enzyme preparations was estimated according to the method of Fishman *et al.* (10). In control experiments the above mentioned enzyme concentrations caused approximately 95% hydrolysis of an estriol glucosiduronate reference standard.

Determination of glucosiduronate glucosiduronic acid.—The method of Fishman and Green (11) was used. The reaction volume was reduced to half of that employed originally by these authors.

Determination of estriol.—The Kober reaction for estriol was carried out according to the specifications of Brown (12), with or without previous hydrolysis of the estriol glucosiduronate.

Acetylation of estriol glucosiduronate.—This was carried out with acetic anhydride in pyridine at room temperature for 24 hours. Excess of acetic anhydride was hydrolyzed by the addition of water. The mixture was poured into a solution of dilute hydrochloric acid containing crushed ice and the acetate was extracted with a mixture of equal volumes of ethyl acetate and ethyl ether. The organic phase was then washed with 5% potassium carbonate and water, dried with sodium sulphate and evaporated to dryness.

Standard preparations.—Two crystalline reference standards of estriol 16α- (or 17β?) (3-D-glucosiduronate) were used; one was extensively purified by ion exchange chromatography,[2] the other was a commercial preparation.[3] These substances behaved as homogeneous compounds, when studied in different systems in countercurrent distribution, paper chromatography and paper electrophoresis. Under our experimental conditions both preparations gave a ratio of glucosiduronic acid to estriol of 1.1.

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[2] This preparation has been obtained through the courtesy of the Late Dr. W. S. Lauld, Montreal, Canada.

[3] Purchased from Sigma Chemical Co., Saint Louis, Mo., U.S.A.

TABLE 1. PARTITION COEFFICIENTS (K) GOVERNING THE DISTRIBUTION OF ESTRIOL GLUCOSIDURONATE IN THE DIFFERENT SOLVENT SYSTEMS USED IN THE EXTRACTION PROCEDURE

All K-values were obtained in 12-transfer countercurrent distributions

Upper phase	Lower phase	K	Step in extraction procedure
n-butanol	water (acidified to pH 2–3)	19.0	3
n-butanol	water (pH 7.0)	2.3	3
n-butanol	0.1N NaOH	0.06	4
n-butanol	1.0N NaOH	0.03	4
petroleum ether	water	0.01	5
ethyl ether	water	0.01	5
water	chloroform	19.0	5
n-hexane:n-butanol (2:1)	water	0.02	7
ethyl acetate	0.1N HCl	1.6	8
benzene:pyridine (2:1)	water	0.01	10
quinoline	10% Na_2CO_3	15.6	11
water	quinoline	0.01	12
0.1N NaOH	quinoline	1.4	13
water	quinoline:carbon tetrachloride (1:1)	3.4	13
n-butanol	glycine buffer (pH 11.0)	1.4	14
n-butanol	1% Na_2CO_3	1.0	16
n-butanol:ethyl acetate (1:1)	0.2% NH_4OH	1.1	16

Extraction procedure.—A method has been evolved for the extraction and purification of estriol glucosiduronate. All steps in this procedure are based on the partition coefficients of estriol glucosiduronate in the different solvent systems.[4] These coefficients are shown in Table 1. The original observations of Cohen and Marrian (13, 14) and those of Bauld (15) were of great value to us in developing the most efficient solvent systems. The present method can also be used for the extraction and purification of small amounts of estriol glucosiduronate from different biological sources; it has been successfully applied so far to pregnancy urine, urine of newborns and to different human tissues. The method is given in outline in Figure 1, whereas some experimental details are given in the form of comments on the different steps of the procedure.

Comments on the extraction procedure:

(1) Approximately 100 gm. of meconium or less are homogenized with 25 gm of ammonium sulphate and 50 gm. of quartz sand and extracted 4 times with 100 ml. of 80% (v/v) ethanol. Following centrifugation the ethanol is kept in the deep-freeze at −17° C for 48 hours and centrifuged again at −14° C. Insoluble residue is discarded.

(2) This is based on the observation of Zander and Simmer (16) that maximal precipitation of fatty material can be achieved by freezing-out at this critical methanol concentration.

(3) The methanol is distilled off under reduced pressure in an atmosphere of nitrogen. It follows from the partition coefficient (Table 1) that butanol extraction can be carried out also at pH 7.0; however, at this pH sometimes troublesome emulsions may arise.

(4) Some earlier workers used N NaOH in this extraction; in view of the virtually identical partition coefficients we prefer 0.1N NaOH.

(5) Ether extraction is necessitated by the fact that (contrary to our expectations) only a part of "free" (unconjugated) estriol is removed by chloroform. The K-value o

[4] The partition coefficient (K) of any solute distributed between two mutually saturated solvents is defined as the concentration in the upper phase divided by the concentration in the lower phase.

estriol between water and chloroform is 0.89. It is of some interest in this connection that between 25% ethanol and chloroform estriol has a K-value of 0.14.

(9) To the ethyl acetate a few drops of conc. ammonium hydroxide are added in order to avoid an acid milieu during evaporation.

(10) Following this partition the aqueous phase is washed once with 1/10th volume of benzene in order to remove traces of pyridine.

(13) In case extraction of estriol glucosiduronate from quinoline at neutrality would be prefered, the quinoline can be diluted with an equal volume of carbon tetrachloride and the organic phase extracted twice with an equal volume of distilled water.

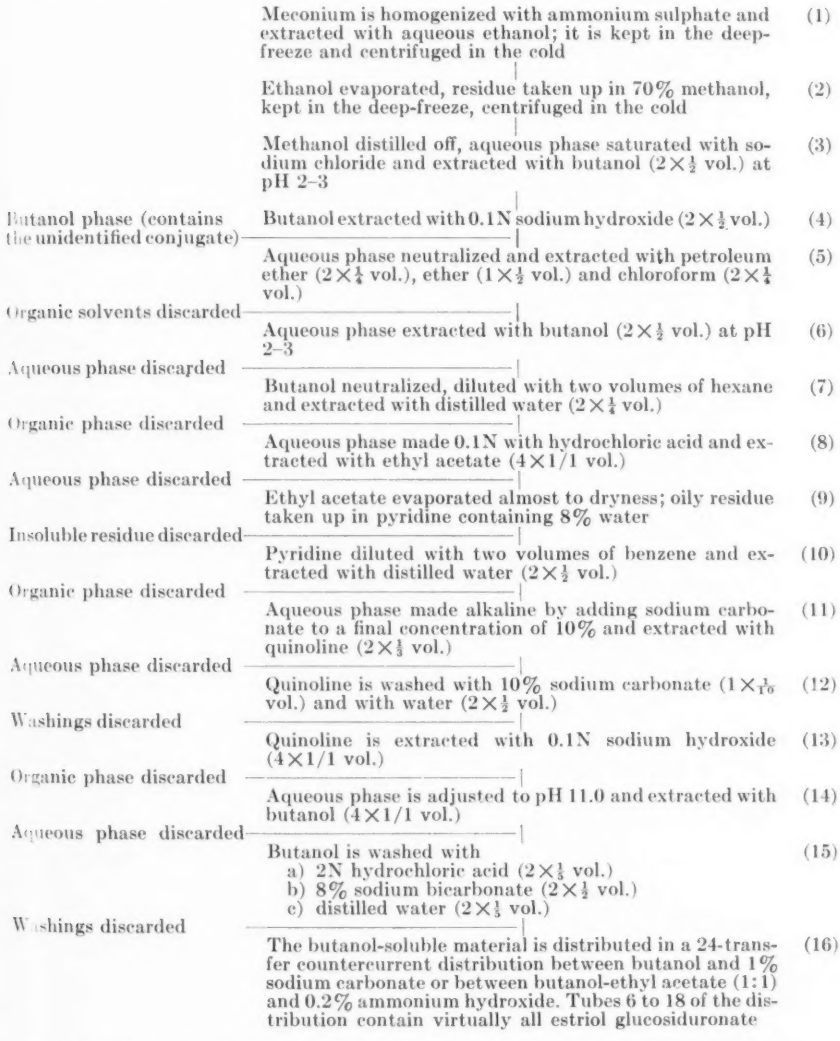
Meconium is homogenized with ammonium sulphate and extracted with aqueous ethanol; it is kept in the deep-freeze and centrifuged in the cold (1)
Ethanol evaporated, residue taken up in 70% methanol, kept in the deep-freeze, centrifuged in the cold (2)
Methanol distilled off, aqueous phase saturated with sodium chloride and extracted with butanol (2×½ vol.) at pH 2–3 (3)
Butanol phase (contains the unidentified conjugate)
Butanol extracted with 0.1N sodium hydroxide (2×½ vol.) (4)
Aqueous phase neutralized and extracted with petroleum ether (2×¼ vol.), ether (1×½ vol.) and chloroform (2×¼ vol.) (5)
Organic solvents discarded
Aqueous phase extracted with butanol (2×½ vol.) at pH 2–3 (6)
Aqueous phase discarded
Butanol neutralized, diluted with two volumes of hexane and extracted with distilled water (2×¼ vol.) (7)
Organic phase discarded
Aqueous phase made 0.1N with hydrochloric acid and extracted with ethyl acetate (4×1/1 vol.) (8)
Aqueous phase discarded
Ethyl acetate evaporated almost to dryness; oily residue taken up in pyridine containing 8% water (9)
Insoluble residue discarded
Pyridine diluted with two volumes of benzene and extracted with distilled water (2×½ vol.) (10)
Organic phase discarded
Aqueous phase made alkaline by adding sodium carbonate to a final concentration of 10% and extracted with quinoline (2×⅓ vol.) (11)
Aqueous phase discarded
Quinoline is washed with 10% sodium carbonate (1×1/10 vol.) and with water (2×½ vol.) (12)
Washings discarded
Quinoline is extracted with 0.1N sodium hydroxide (4×1/1 vol.) (13)
Organic phase discarded
Aqueous phase is adjusted to pH 11.0 and extracted with butanol (4×1/1 vol.) (14)
Aqueous phase discarded
Butanol is washed with (15)
a) 2N hydrochloric acid (2×⅕ vol.)
b) 8% sodium bicarbonate (2×½ vol.)
c) distilled water (2×⅓ vol.)
Washings discarded
The butanol-soluble material is distributed in a 24-transfer countercurrent distribution between butanol and 1% sodium carbonate or between butanol-ethyl acetate (1:1) and 0.2% ammonium hydroxide. Tubes 6 to 18 of the distribution contain virtually all estriol glucosiduronate (16)

FIG. 1. Scheme for the isolation of estriol glucosiduronate from human meconium.

TABLE 2. COMPARISON OF Rf VALUES OBTAINED WITH AN ESTRIOL GLUCOSIDURONATE REFERENCE STANDARD AND WITH AN ESTRIOL GLUCOSIDURONATE-LIKE COMPOUND ISOLATED FROM EXTRACTS OF HUMAN MECONIUM

System	Solvent composition	Parts (ml.)	Source (Reference)	R_f	
				Reference compound	Extract of meconium
1	toluene tert-butanol acetic acid water	150 50 60 140	(17)	0.02	0.02
2	ethyl acetate n-hexane acetic acid water	120 80 60 140	(18)	0.17	0.17
3	n-butyl ether n-butanol acetic acid water	130 70 60 140	(18)	0.25	0.25
4	n-butanol tert-butanol 2N NH_4OH	150 50 200	(17)	0.71	0.69
5	ethylene dichloride tert-butanol acetic acid water	150 50 60 140	(17)	0.77	0.77
6	n-butanol acetic-acid water	200 20 180	(*)	0.86	0.86

(*) Present investigation.

(14) The pH can be adjusted for instance by the addition of glycine, sodium bicarbonate or ammonium sulphate.

Yield of estriol glucosiduronate.—In five experiments on different pools of meconium the yield varied between 0.5 and 1.8 mg. per 100 gm. meconium with an average of 1.0 mg. per 100 gm.

Characterization of the isolated estriol glucosiduronate-like material.

Paper partition chromatography.—The partition characteristics of the isolated material were compared with those of the reference compound in six different solvent systems. The Rf values obtained are compiled in Table 2. It appears from the data of Table 2 that the isolated material shows the same Rf values as the reference standard both in alkaline and acid systems. Furthermore, since the spots could be directly visualized following spraying with reagents for phenols, it can be concluded that the phenolic hydroxyl group of estriol was free. It is not yet known whether or not the systems employed in this study are suitable for the separation of the glucosiduronates of different steroids. However, in preliminary experiments, using solvent system No. 4 (see Table 2) it was possible to separate

TABLE 3. PAPER ELECTROPHORETIC MOBILITIES IN DIFFERENT BUFFER SOLUTIONS OF ESTRIOL GLUCOSIDURONATE REFERENCE STANDARD AND OF THE ESTRIOL GLUCOSIDURONATE-LIKE SUBSTANCE ISOLATED FROM HUMAN MECONIUM

Potential: 300 volts. Current: 8–10 mA. Running time: 16 hours

Buffer	pH	Migration (cm)	
		Reference compound	Extract of meconium
Phosphate	6.8	8.1	8.0
Veronal	8.6	10.0	10.0

estriol glucosiduronate completely from 17β-estradiol-17-glucosiduronate,[5] whereas the glucosiduronates of testosterone[5] and of dehydroepiandrosterone[5] exhibited Rf values similar to that of estriol glucosiduronate.

Paper electrophoresis.—The results of these experiments are shown in Table 3. It appears from the data that in two different buffers at different pH values the reference standard preparation and the meconial extract migrate at identical rates. Furthermore, preliminary experiments indicate that paper electrophoresis in veronal buffer effectively separates the glucosiduronates of estriol, 17β-estradiol, testosterone and dehydroepiandrosterone. Thus it would appear that paper electrophoresis is a helpful tool in separating of steroid glucosiduronates of different types.

Countercurrent distribution.—The partition coefficients obtained in 8 solvent systems are summarized in Table 4. The data of Table 4 indicate that in all solvent systems investigated identical K values were obtained for the reference standard and for the substance isolated from meconium. Furthermore, when the extract obtained from meconium was distributed in a 24-transfer countercurrent distribution and the contents of each tube were analyzed simultaneously both for estriol and for glucosiduronate glucosiduronic acid, these two parameters indicated virtually identical results, as shown in Figure 2. The molar ratio of estriol to glucosiduronic acid was calculated to be 1:1.1–1.2, when the top tubes of the distribution were analyzed. Also this fact suggests that one is dealing here with an estriol monoglucosiduronate.

Following acetylation of the purified meconial extract the products of acetylation were partitioned in a countercurrent distribution. The results are shown in Figure 3. It can be seen that 2 components are separated following the distribution, one with a K value of 0.02, which is believed to be the non-acetylated compound, and another component with a K value of 10.1. Such a high partition coefficient in this system is consistent with the view that one is dealing with an acetylated estriol glucosiduronate. The same partition coefficients were obtained when the acetylated standard preparation was distributed in this system.

[5] We are indebted to Dr. H. H. Wotiz, Boston, U.S.A. for a generous gift of these steroid glucosiduronates.

TABLE 4. PARTITION COEFFICIENTS IN DIFFERENT SOLVENT SYSTEMS OF ESTRIOL GLUCOSIDURONATE REFERENCE STANDARD AND OF THE ESTRIOL GLUCOSIDURONATE-LIKE SUBSTANCE ISOLATED FROM HUMAN MECONIUM

All K values were obtained in 24-transfer countercurrent distributions

Solvent system				Partition coefficient (K)	
Upper phase	ml	Lower phase	ml	Reference compound	Extract of meconium
n-butanol tert-butanol	375 125	2N NH_4OH	500[1]	0.69	0.68
n-butanol ethyl acetate n-hexane	300 100 100	water	500	0.69	0.64
n-butanol ethyl acetate	250 250	0.2% NH_4OH	500[2]	1.1	1.2
0.1N NaOH	500	quinoline	500	1.3	1.4
ethyl acetate	500	0.1N HCl	500	1.6	1.6
n-butanol	500	0.8% Na_2CO_3	500	1.4	1.3
n-butanol	500	water	500	2.3	2.3
n-butanol ethyl acetate	350 150	10% NaCl	500	3.2	3.4
Acetylation product					
n-hexane benzene	400 100	methanol water	250 250	10.1	10.1

[1] This system was described for paper chromatography by Bush (17).
[2] This system was developed by Schneider & Lewbart (18).

In order to demonstrate that the estrogen part of the conjugate is indeed identical with estriol, the following evidence has been accumulated after acid-hydrolysis of the estriol glucosiduronate-like material:

a) the Rf values were identical with those of authentic estriol in two solvent systems

b) the partition coefficients in two countercurrent distributions agreed with those of estriol

c) following methylation of the phenolic hydroxyl group the chromatographic behavior on alumina was the same as that of estriol 3-methyl ether

d) the infrared absorption spectrum of the methylated compound agreed with that of estriol 3-methyl ether.[6]

Enzyme hydrolysis.—When 13.8 μg. estriol equivalent of the meconial estriol glucosiduronate-like material was incubated with bacterial β-glucuronidase under the conditions described, 94% of the estriol was recovered as "free" estriol. When 14.9 μg. estriol equivalent was incubated

[6] We are indebted to Dipl. Eng. S. Veige (AB Leo, Hälsingborg) for carrying out the infrared spectrographic identification.

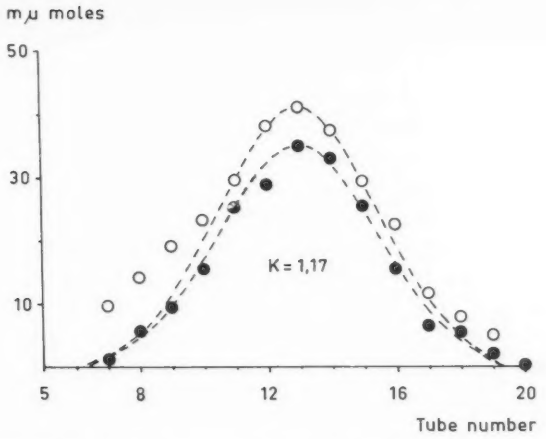

FIG. 2. Countercurrent distribution of an estriol glucosiduronate-like compound isolated from extracts of human meconium. Solvent system: 50% n-butanol +50% ethyl acetate (upper phase) and 0.2% ammonium hydroxide (lower phase). Filled circles indicate the results of the estriol determinations, open circles those of the glucosiduronate glucosiduronic acid determinations. *K* is the partition coefficient calculated according to Way and Bennett (8). The same partition coefficient was calculated from both curves.

with the Helix pomatia enzyme, 95% "free" estriol was found. This was in contrast to the findings on the unidentified "butanol-soluble, alkali-insoluble" estriol conjugate (separated in step 4 in Fig. 1) which was not hydrolyzable by the bacterial β-glucuronidase, but was almost completely

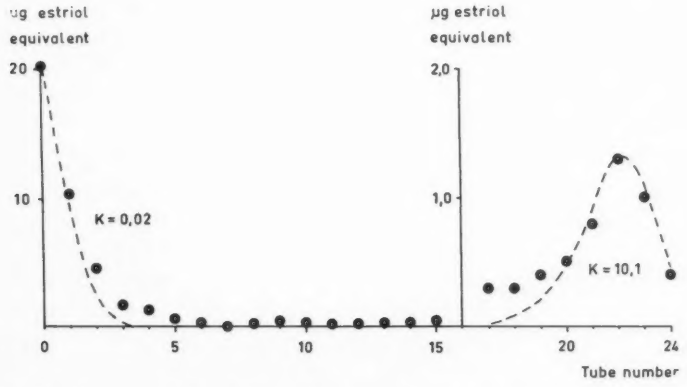

FIG. 3. Countercurrent distribution of the products obtained following acetylation of an estriol glucosiduronate-like compound isolated from human meconium. Solvent system: 80% n-hexane+20% benzene (upper phase) and 50% methanol (lower phase). *K* is the partition coefficient.

(92%) hydrolyzed by the Helix pomatia preparation, which contains a considerable sulphatase activity.

Attempts to characterize the "butanol-soluble, alkali-insoluble" conjugate.— This conjugate could not be extracted from butanol with N sodium hydroxide. Following countercurrent distribution in the system: 75% n-butanol+25% tertiary butanol (upper phase) and 2N ammonium hydroxide (lower phase) this compound distributed itself with a K value of 2.3, whereas estriol glucosiduronate in the same system exhibited a value of 0.68. When the "butanol-soluble, alkali-insoluble" material was partitioned in another solvent system consisting of 50% n-butanol+50% ethyl acetate (upper phase) and 0.2% ammonium hydroxide (lower phase) a K value of 1.9 was obtained, whereas estriol glucosiduronate exhibited a constant of 1.1. These data together with the results of hydrolysis experiments with different enzymes suggest that this material might be an estriol sulphate. Further work on the characterization of this conjugate is in progress now in this laboratory.[7]

DISCUSSION

The view that the material isolated from meconium is identical with estriol glucosiduronate is based on the following evidence:

1) Agreement of Rf values in 6 different solvent systems
2) Identical electrophoretic mobilities at two pH-values
3) Agreement of partition coefficients in 8 different solvent systems
4) Agreement of partition coefficients following acetylation
5) Agreement of the K values estimated as glucosiduronate glucosiduronic acid and as estriol, respectively, following countercurrent distribution. Identical molar ratios of estriol to glucosiduronic acid in meconial extract and standard preparation, respectively
6) Complete hydrolysis of the compound by a bacterial β-glucuronidase preparation
7) Identification of estriol as the estrogen part of the conjugate.

In view of this accumulated evidence it is concluded that the material isolated from the meconium of male newborns is identical with estriol glucosiduronate.

The presence of huge amounts of estriol glucosiduronate in meconium is of considerable physiological interest, since the placento-amniotic barrier does not seem to be permeable to this compound to any major extent (cf. 19). Thus the present findings raise the question as to the possible existence of a fetal glucosidurono-conjugating mechanism. It has been shown re-

[7] While this paper was in press, the identity of this conjugate with estriol-3-sulphate (sodium salt) could definitely be established. Full details of the identification will be published in a forthcoming paper.

cently that human fetuses in the 4th month of gestation are capable of conjugating intra-amniotically administered estriol, even in case the fetal organism has been separated from the maternal one prior to the injection (20). The conjugated estriol found in the lungs and livers of such fetuses can, however, not be extracted from butanol with N sodium hydroxide, which suggests that—most probably—it is not identical with a glucosiduronate. On the other hand we have found recently considerable amounts of estriol glucosiduronate-like material in the contents of the gastrointestinal tract of fetuses of the same age (Menini and Diczfalusy, unpublished observations). Thus it could be suggested—very tentatively—that in the human fetus (perhaps also in the adult?) two estrogen-conjugating mechanisms are operating: an initial, rapid one (perhaps sulpho-conjugation?) functioning mainly in different tissues such as lungs, liver, etc., and a second one, a glucosiduronate-conjugating mechanism, located mainly in the gastrointestinal tract and in the liver. Although this may sound highly speculative, it should be remembered that Hartiala and co-workers (21) recently succeeded in demonstrating the formation of 17β-estradiol glucosiduronate following the incubation of rat intestinal mucosa with 17β-estradiol *in vitro*. Moreover, at abdominal operations in the human Diczfalusy *et al.* (22) recently demonstrated that a large part of the estriol, which was injected into an intestinal loop, could be recovered from the intestinal wall and from the effluent venous blood in a "conjugated" form. One component in this conjugated material was identified as estriol glucosiduronate. Since hardly any conjugated material remained in the butanol phase following extraction with N sodium hydroxide, it seems unlikely, that under the experimental conditions used greater amounts of estriol sulphate would be formed in the human small intestine. At any rate, it appears that the small intestine in the adult human subject is capable of conjugating estriol and that at least a part of this conjugated estriol is identical with estriol glucosiduronate. Thus one of the questions to be answered at present is, whether or not the intestinal mucosa of the human fetus in the 2nd trimester of gestation is already able to conjugate estriol with glucosiduronic acid, or whether the estriol glucosiduronate found in the intestinal tract of fetuses is formed elsewhere. *In vitro* incubation studies as well as the oral administration of estrogens to previable fetuses might perhaps provide an answer to this question. Such experiments are now in progress in this laboratory.

Acknowledgment

We are indebted to the late Dr. W. S. Bauld (Montreal) for a gift of crystalline estriol glucosiduronate and to Dr. H. H. Wotiz (Boston) for a gift of 17β-estradiol glucosiduronate, testosterone glucosiduronate and dehydroepiandrosterone glucosiduronate. We also wish to thank Dipl. Eng. S. Veige (AB Leo, Hälsingborg) for carrying out the infrared spectrographic identification and Miss Berit Martinsen for her co-operation in some of the experiments.

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RETARDATION OF SEXUAL DEVELOPMENT IN FEMALE RATS DUE TO IPRONIAZID TREATMENT

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ABSTRACT

Prolonged oral administration of iproniazid dihydrochloride to female rats, in daily doses of 53–224 mg./kg., causes alterations in the sexual function and in the hypophysis. The drug retards sexual maturation, lengthens the estrous cycle, inhibits corpus luteum formation, leads to the appearance of "deficiency cells" and hypotrophy of the ovarian interstitial tissue.

Retarded uterine development is also observable. All these changes are transient and probably due to a partial blockade of ICSH secretion.

THE most widely accepted interpretation of the therapeutic activities of iproniazid, the psycho-energising and the anti-anginal, is based on the mono-amine-oxidase blocking activity of this drug with the consequent accumulation of 5-hydroxy-tryptamine in the nervous tissue and of catecholamines in the cardiac muscle (1).

Although there are many reasons to object to this theory (2, 3), researches on animals failed up to now to find a clue to a better understanding of the mechanism of the therapeutic action of iproniazid, probably because they were mainly conducted on an acute basis whereas it is well known that the drug must be administered many days before taking effect on humans.

Therefore we planned a research of prolonged administration of the drug, concentrating our attention upon the endocrine system. The present report describes some of the changes produced in the hypophysis and ovaries of rats by a chronic iproniazid treatment.

METHODS

Iproniazid was administered orally to different groups of rats of both sexes and various ages. The drug was mixed at a known ratio with the animals' food. Everyday the food given to the animals, the discarded food and the food left were weighed. By allowing for these differences the daily food and drug intake was calculated. The animals were weighed at the beginning, during and at the end of the test. Blood counts and pictures for every animal were taken in the course of and at the end of the experiment. The perineal region of the females was inspected daily for patency of the vagina, regarded as an index of sexual maturation. The percentages of sexually mature females, recorded daily, were transferred to logarithmic-probability paper and the time in days from the

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beginning of the experiment to the patency of the vagina in 50% of the animals was calculated by the Litchfield (4) method. After sexual maturation vaginal smears were taken daily in order to determine the phase of the estrous cycle. Finally, the animals were killed by ether-chloroform vapours, and the genital and other important organs were weighed and examined histologically after H-E stain. The hypophysis was stained according to the Mallory-type panoptic method of Rasmussen-Ignesti (5).

To test the gonadotrophic activity of the pituitaries of the rats, each gland was finely and separately homogenised in a heavy test tube with the aid of a ground glass rod and then suspended in 0.6 ml. isotonic saline solution and kept at −20° C. This suspension was injected subcutaneously in portions of 0.2 ml. into immature female mice for 3 days. On the fourth day the mice were killed and the uteri and ovaries weighed.

The direct effect of iproniazid upon the uterus and any possible interference with the estrogenic hormones was tested by the method described by Velardo and Sturgis (6) and by Setnikar *et al.* (7) in female rats ovariectomised at the age of 80 days. One week after spaying the drug was injected subcutaneously for three days, alone or with 0.5 μg. estradiol benzoate dissolved in 0.5 ml. olive oil. The hormonal treatment causes a marked increase in the weight of the uterus due to imbibition and cell proliferation (8).

The acute LD50s quoted were calculated by the Litchfield and Wilcoxon method (9) on the basis of the mortality rate observed during the course of 5 days from a single acute drug administration.

Iproniazid dihydrochloride was used throughout these experiments and the dosages given refer to this substance. The molecular weight ratio of the dihydrochloride salt to the iproniazid base is about 1:0.7.

RESULTS

Effects of prolonged administration of iproniazid on the sex organs. Four experiments in chronic oral administration of iproniazid mixed with food were performed. In the first iproniazid was mixed with the food at the ratio of 0.05%, in the other three the ratio was 0.172%. The average dose of iproniazid ingested daily by the animals is given in Table 1, which shows also the initial average weights and ages of the animals and the duration of each experiment. As in the male rats no statistically significant ponderal or histological changes in the hypophysis or in the sex organs (testicles, seminal vesicles and prostates) were observed, only the principal results for the females are reported.

The most striking changes were seen on Experiment 2, *i.e.* after a daily administration of 224 mg./kg. iproniazid for 30 days. The normal development of the ovaries, uterus and hypophysis was impeded and noteworthy histological changes were found in the uterus and ovaries.

In the treated animals the uterine wall (Fig. 1a) was less developed and the muscle cells were smaller and uncrowded. The uterine epithelium was very regular, cylindrical, with nuclei lying almost all at the same level and with faintly basophil cytoplasm. Histologically the uterus was in every aspect comparable to that of the infantile uterus, whereas in the control rats (Fig. 1b) the uterine wall showed not only a far more developed muscular and stromatic component but also an intensely active mucosa (crowded cells with nuclei disposed in several layers, intense basophilia of the cytoplasm).

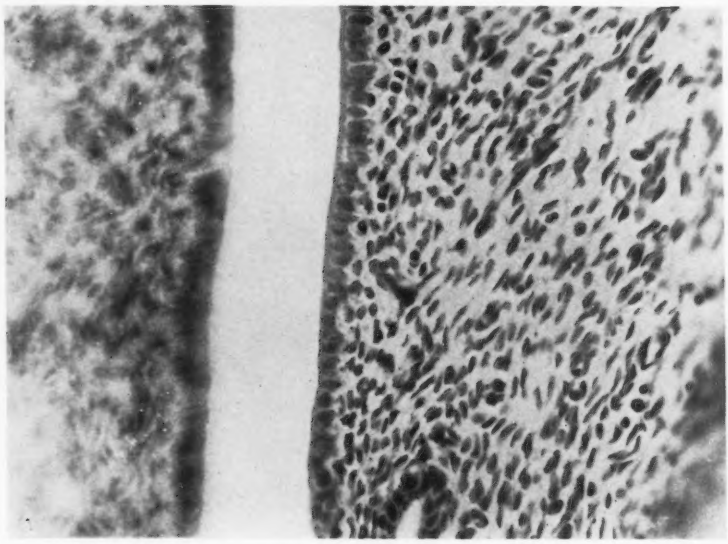

FIG. 1a. Histological picture of the uterus of a rat treated for 30 days with 224 mg./kg. iproniazid. The organ has conserved its infantile appearance. The epithelial cells of the mucosa are devoid of the cytoplasmic granules and show small thinly distributed nuclei, having low chromatic content (H-E staining, magnification ×400).

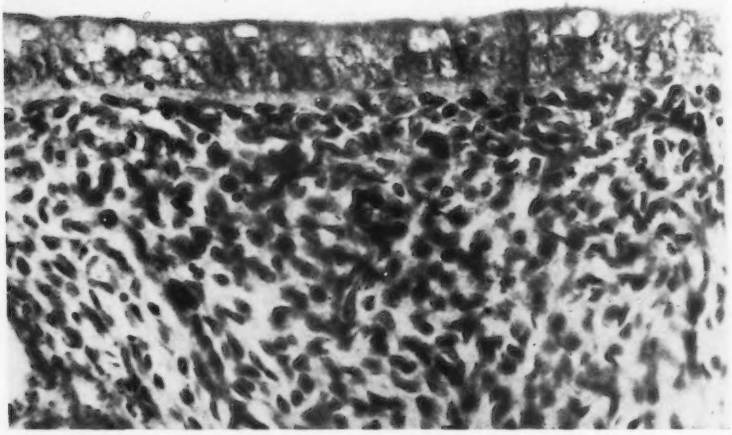

FIG. 1b. Uterus of control rat, staining and magnification as in Fig. 1a.

The epithelial cells of the mucosa are thickly distributed, the cytoplasm contains basophil granules and vacuoles, and the nuclei, which are larger than those of Fig. 2, contain a greater quantity of chromatin collected in the granules.

FIG. 2. Ovary of a rat treated for 30 days with 224 mg./kg. iproniazid. Note the absence of corpora lutea, the reduction of interstitial tissue and the presence of "deficiency cells." The development and the number of follicles are normal (×30).

In the ovaries (Figs. 2 and 3) the interstitial tissue was much less developed in the treated animals than in the controls. The chromatin of the nuclei of the interstitial cells showed a tendency to agmination as after hypophysectomy so that these cells resembled the "deficiency cells" described by Selye *et al.* (10). Further, while in the controls the corpora lutea could be detected in all the sections, they were extremely rare in the treated animals, so much so that these formations could only be found in a very small number of the serial sections. Most follicles in the treated animals were small or medium-sized; a few matured further with ovocytes free in well-developed antra.

This delay in the development of the ovaries and uteri, though less evident, was nonetheless noticeable on a weight and histological basis also in the other two Experiments, the 3rd and 4th of Table 1, in which the animals were given the same daily doses of iproniazid, (about 200 mg./kg.), but over a longer period, *viz.* 40 and 67 days respectively.

In Experiment 1 in which the animals were given for 61 days a much smaller dose of iproniazid (53 mg./kg. daily), the ponderal and histological alterations were absent.

Effects of iproniazid upon the estrous cycle. The retarding influence of iproniazid upon the sexual maturation of the females was striking: the treated rats matured 6–7 days later than the controls (Table 1, Experi-

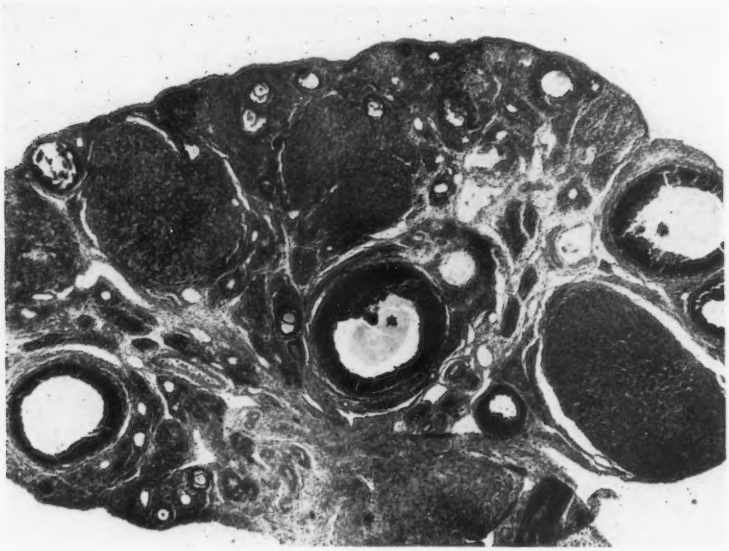

FIG. 3. Histological picture of a control ovary.

ments 1, 3 and 4). This phenomenon was observed also at the dose of 53 mg./kg. daily.

The course of estrus was profoundly modified by iproniazid, as can be seen particularly well in the animals treated for a long time, for example in Experiments 1 and 4. The first two-three cycles were much more irregular in the treated animals and in every case lasted much longer than in the

TABLE 1. EFFECTS OF A PROLONGED IPRONIAZID TREATMENT UPON FEMALE RATS

Experiment No.	1		2		3		4	
Duration of the Experiment	61 days		30 days		40 days		67 days	
Average age of the rats at the beginning of treatment	38 days		45 days		50 days		35 days	
Treatment	Controls	Iproniazid 53 mg./kg. daily	Controls	Iproniazid 224 mg./kg. daily	Controls	Iproniazid 205 mg./kg. daily	Controls	Iproniazid 200 mg./kg. daily
No. of animals	10	10	10	10	20	20	10	10
Daily food consumption gm./kg.	113.0	106.0	126.0	130.0	121.0	122.0	119.0	121.0
Initial weight (gm.)	61.1±1.6	60.9±1.5	84.7±1.3	85.3±1.2	107±2	106±2	51.1±2.3	50.9±2.3
Final weight (gm.)	199±11	192±11	155±4	106±2 *<0.001*	170±5	137±4 *<0.001*	199±8	169±4 *0.01–0.001*
Ovaries mg./100 gm. bodyweight	51±2	54±2	49±2	37±4 *0.02–0.01*	59±3	51±4	53±2	55±4
Uterus mg./100 gm. bodyweight	156±6	166±21	156±15	48±11 *<0.001*	151±9	79±8 *<0.001*	150±10	114±13 *0.05–0.02*
Hypophysis mg./100 gm. bodyweight	3.82±0.75	3.62±0.37	5.16±0.37	3.51±0.57 *0.05–0.02*	5.59±0.30	4.81±0.14 *0.02–0.01*	4.59±0.19	4.39±0.11
Vagina patency ED50 days after beginning of treatment	24±2*	31±2* *<0.05*			10±2*	16±3* *<0.05*	22±1*	28±1* *<0.05*
Duration of estrous cycle (days)	4.3±0.2	6.2±0.5 *0.01–0.001*					5.2±0.3	7.5±0.6 *0.01–0.001*

When the difference between control and treated rats is statistically significant, *i.e.* when the P of Student's t <0.05, the P value is given in italics.

* P=0.05 confidence limits.

TABLE 2. EFFECTS OF IPRONIAZID UPON THE DURATION OF THE ESTROUS CYCLE (DAYS)

Dose	53 mg./kg. daily (Exp. 1, Table 1)			200 mg./kg. daily (Exp. 4, Table 1)		
Treatment	Control	Treated	P_t difference	Control	Treated	P_t difference
1st cycle	4.6±0.5	8.8±1.2	0.01−0.001	5.8±0.5	9.9±1.4	0.01−0.001
2nd cycle	3.8±0.4	7.5±1.4	0.05−0.02	4.8±0.5	8.4±0.5	0.01−0.001
3rd cycle	4.0±0.3	6.2±0.7	0.02±0.01	5.0±0.6	6.7±0.7	
4th cycle	4.7±0.3	5.8±1.3		5.0±0.7	5.1±0.8	
5th cycle	4.1±0.3	4.3±0.6				
6th cycle	4.3±0.3	4.6±0.5				
Average duration	4.3±0.2	6.2±0.5	0.01−0.001	5.2±0.3	7.5±0.6	0.01−0.001

controls because of a longer diestrus phase. The average duration of the cycles was considerably and significantly longer in the treated animals than in the controls. The duration of the estrous cycle seemed to return almost to normal after the 3rd and 4th cycle (Table 2).

Effects of iproniazid upon the weight and histological aspect of the hypophysis. The weight of the pituitaries of the rats treated with iproniazid was in every case less than that of the controls. This difference attained statistically significant levels in the females after treatment with high doses (224 and 205 mg./kg. daily) for 30 and 40 days respectively (Table 1, Experiments 2 and 3). However no substantial histological difference was detectable, and no difference in the ratios of acidophil to basophil cells between control and treated animals was found (Table 3). Also the pituitaries of the males, which were subjected to the same iproniazid treatment as the females, did not show any shift in the ratios of acidophil to basophil cells (Table 3).

Action of iproniazid upon the gonadotrophic hormone content of the hypophysis. The effects of injecting the pituitaries of rats subjected to a daily oral treatment with 205 mg./kg. iproniazid for 40 days (Experiment 3, Table 1) into impuberal female mice, were as follows:

A) None upon bodyweight (Table 4).

B) The pituitaries of the rats treated with iproniazid seemed to contain greater gonadotrophic activity than that of the controls as shown evidently by the weights of the uteri and ovaries of the mice treated with hypophysis of female rats. When it is borne in mind that the weight of the female rat pituitaries treated with iproniazid was on

TABLE 3. RATIO OF ACIDOPHIL TO BASOPHIL CELLS IN THE PITUITARIES OF THE RATS OF EXPERIMENT 2 (TABLE 1)

	Males	Females
Controls	2.9±0.3	2.1±0.3
Treated	3.0±0.6	2.8±0.7

TABLE 4. EFFECTS OF IPRONIAZID UPON THE GONADOTROPHIC ACTIVITY OF THE PITUITARIES IN THE RATS OF EXPERIMENT 3 (TABLE 1) AS TESTED ON IMMATURE MICE

Treatment	No. of mice	Bodyweight gm.	Uterus mg./100 gm. body wt.	Ovaries mg./100 gm. body wt.
Saline solution	15	11.4 ± 0.4	87 ± 0.4	65 ± 4
Pituitaries of control males	9	13.1 ± 0.5*	268 ± 20	154 ± 10
Pituitaries of male treated with iproniazid†	8	11.6 ± 0.4	286 ± 32	144 ± 9
Pituitaries of control females	8	11.0 ± 0.6	199 ± 15 (P_t = 0.05 − 0.02)	84 ± 3 (P_t > 0.05)
Pituitaries of females treated with iproniazid	7	11.0 ± 0.4	284 ± 36 (P_t = 0.05 − 0.02)	94 ± 5 (P_t > 0.05)

* The bodyweight of these animals was above the average before the beginning of the experiment.

† These animals were subjected to the same iproniazid treatment as the females (Experiment 3, Table 1).

average 6.63 ± 0.31 mg. while the corresponding figure for the female controls was 9.39 ± 0.48 mg., it must be concluded that the content of gonadotrophic activity per unit of weight was considerably increased by iproniazid treatment. No such difference in the gonadotrophic content of the pituitaries of the male control rats and of those of the iproniazid treated was found.

Pro- and anti-estrogenic action. In the ovariectomized rats iproniazid, administered in daily doses of 6 to 48 mg./kg. for 3 days led to no statistically significant variations in the weight of the uterus. As the intraperitoneal LD50 proved to be equivalent to 245 mg./kg., with confidence limits (P = 0.05) from 312 to 193 mg./kg., the daily doses administered ranged from about 1/40 to 1/5 of this LD50. These doses, given in association with a daily dose of 0.5 μg. estradiol benzoate, enhanced the uterotrophic effect of this hormone, Table 5, whereas in experiments in which the same doses of iproniazid were tested on rats treated with minimal effective doses of estradiol (0.015 μg.) no significant influence on the estradiol-induced increase in uterus weight was found.

TABLE 5. EFFECTS OF IPRONIAZID UPON THE ESTROGENIC ACTIVITY OF ESTRADIOL

Daily treatment (for 3 days)	No. of animals	Uterus mg./100 gm. body weight	% difference and P_t diff.	Comparison group
1. Controls	7	16 ± 1		
2. Estradiol 0.5 μg. s.c.	10	64 ± 4	+300% (<0.001)	1
3. Estradiol s.c. + Iproniazid 6 mg./kg. s.c.	10	68 ± 5	+ 6% (>0.05)	2
4. Estradiol s.c. + Iproniazid 12 mg./kg. s.c.	9	92 ± 11	+ 44% (0.02 − 0.01)	2
5. Estradiol s.c. + Iproniazid 24 mg./kg. s.c.	4	84 ± 5	+ 31% (0.01 − 0.001)	2
6. Estradiol s.c. + Iproniazid 48 mg./kg. s.c.	7	76 ± 5	+ 19% (>0.05)	2

Discussion

The results show that prolonged oral administration of elevated doses of iproniazid retards the sexual maturation of female rats, prolongs the quiescent period of the estrous cycle, slows down the normal development of the uterus and leads to hypotrophy of ovarian interstitial tissue, the cells of which tend to assume the appearance of "deficiency cells."

The first point to be discussed is whether these effects of iproniazid upon the sexual development of the rats (detectable only in females) are to be included among the toxic manifestations due to elevated dosage or whether they are an evidence of a more specific pharmacological action. There is no doubt that a daily dose of over 200 mg./kg., which corresponds to about 1/7th of 1470 mg./kg. *i.e.* the acute oral LD50 ($P=0.05$ confidence limits from 1470 to 1749 mg./kg.), as administered in Experiments 2, 3 and 4 (Table 1), gives rise to general toxic manifestations. Indeed these animals showed slower increase in bodyweight, a reduction in hemoglobin and in red cells, a weight loss in the adrenals and a weight gain in the spleen with the typical manifestations of hemosiderosis similar to those described by Benson *et al.* (11). On the other hand as both groups of animals, the treated as well as the controls, ate the same quantity of food per unit of body weight (Table 1), it is unsafe to ascribe the observed impairment of body weight in the treated animals and the observed differences in the weights of the organs solely to possible inanition. Besides the retarding of sexual maturation showed itself, however, just as clearly in the rats treated with 53 mg./kg. daily, *i.e.* with about 1/30 of the acute oral LD50, and no other toxic phenomena were noted in these animals. The retardative effect upon sexual maturation may therefore represent a pharmacological action because it can be demonstrated even with relatively low doses of the drug.

This effect probably does not manifest itself at the periphery, as in the experiment covered by Table 7 no inhibition of the uterotrophic activity of estradiol by iproniazid could be demonstrated.

On the histological evidence, it seems more likely that iproniazid interferes with the secretion of the estrogenic hormones by the ovaries or more precisely with the gonadotrophic stimulus, for the hypotrophy of the interstitial tissue of the ovaries, the presence of "deficiency cells" and the absence of corpora lutea all evince a hypophyseal deficiency.

The failure of iproniazid to influence the sexual development of male may be explained by the observations of Greep *et al.* (12), who found that the interstitial cells of hypophysectomized female rats are much less sensitive to exogenous ICSH stimulation than those of males.

If this finding can be extended to endogenous ICSH, partial inhibition of this hormone by iproniazid may be sufficient to affect sexual maturation in the females whereas in the males, owing to the greater sensitivity to this hormone, no such effect may be shown.

Table 4 shows that iproniazid does not reduce the gonadotrophic content of the pituitaries of rats, but in the case of females, it seems actually to increase it.

It would therefore seem that iproniazid does not decrease ICSH formation by the hypophysis but rather that it blocks the secretion or the peripheral utilization of the hormone by the ovaries.

This blocking action exerted by iproniazid is not permanent; indeed whereas the first sex cycles were considerably disturbed, they became regular after 3–4 weeks. Uterine infantilism also was less pronounced the

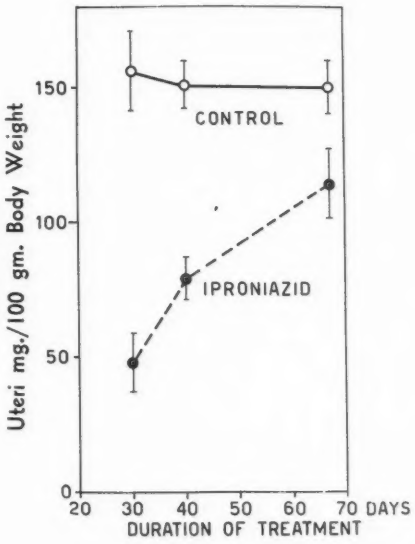

FIG. 4. Weight of uterus (expressed in mg. per 100 gm. bodyweight) in terms of the duration of treatment with about 200 mg./kg. iproniazid by oral route. The more treatment is prolonged the closer the uterine weight gets to normal values.

longer the treatment went on, that is, the more time passed from sexual maturation (Fig. 4). Thus, if more than a month is allowed to pass between sexual maturation and killing, as was done in the case of Experiments 1 and 4 (Table 1), the ponderal and histological alterations detectable in the uterus and ovaries were very slight, although sexual development was certainly retarded also in these Experiments, as shown by the delay in the opening of the vagina and the irregularity of the initial estrous cycles (Tables 1 and 2). For the same reason the maximum retardations of sexual development was encountered in Experiment 2 (Table 1), where the rats were killed a few days after maturation.

It is certainly not easy to establish whether the antigonadotrophic activity of iproniazid described in this paper is of any practical importance, that is, whether it can be used therapeutically, whether it can explain, at least partially, the drug's anti-anginal action or whether it may be responsible for some of the side-effects observable with iproniazid therapy.

It is quite true that sexual side-effects, especially impotence, often occur in the course of iproniazid treatment with 1–3 mg./kg. daily doses, *i.e.* at much lower doses than those used in our experiments. But it is very difficult in our present state of knowledge to attribute this symptom directly to the antigonadotrophic effect of the drug. At all events, this aspect should not be forgotten, especially when iproniazid is prescribed to sexually underdeveloped persons or in the course of sexual maturation.

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BIOLOGICAL ACTIVITIES OF SOME STEROID ETHERS: METHYLTESTOSTERONE ENOL ETHERS

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ABSTRACT

In order to determine the effect on biological activity (in the rat) brought about by the conversion of the α,β-unsaturated carbonyl group to an enol ether, a variety of methyltestosterone ethers were assayed, orally and parenterally, with respect to androgenic and myogenic activity. The enol ethers were found, rather surprisingly, more active than the parent ketone when given by mouth, but less active when injected. The influence upon activity by the character of alcoholic chain is discussed.

IN A previous communication on the preparation of new enol ethers of Δ^4-3-ketosteroid hormones (1) it was anticipated that many of these compounds would be more active than the parent steroids when administered by oral route, but less active when administered parenterally. In this paper and in the forthcoming ones we shall give a full report of these studies; at the same time we shall attempt to explain the observed behavior, which is not only seen in the enol ethers of Δ^4-3-ketosteroids but also in normal 3-O-ethers (2). Besides pointing out differences in the degree of activity, we shall also aim at emphasizing the differences in the pattern of activity which may occur between ethers and parent compounds.

Strangely enough, the biological activity of steroidal enol ethers, which have been known for over twenty years (3) as intermediates in the synthesis of various steroid hormones, has so far escaped the attention of researchers despite the keen search for new and more potent hormonal compounds. It is true that we find in some earlier patents of Schering A.G. (4) mention of a high and prolonged action on the capon comb by parenterally administered benzyl and cyclohexyl enol ethers of testosterone. However, in a more recent study by Junkmann (5), only a very weak activity was observed in compounds of this type, administered by the same route.

In the last few years we have prepared a considerable number of enol ethers by condensing various aliphatic, cycloaliphatic and arylaliphatic alcohols with androstenedione, testosterone and the 17-esters of the latter (1). All these compounds showed little or no activity (weight of seminal vesicles, ventral prostate, levator ani of the castrated rat) when admin-

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istered subcutaneously (6, 7), though some of them proved to be useful as palliatives in human mammary cancer (8, 9). When the above compounds were administered orally, they did not differ appreciably in activity from their respective parent compounds administered the same way (10). Therefore it was surprising that a potentiation of activity was observed with the enol ethers of methyltestosterone when compared with methyltestosterone itself.

MATERIAL AND METHODS

Male albino rats from our stock, weighing about 50 gm. and maintained on a standard diet, were castrated under ether anesthesia. The assays were begun on the following day and extended over a period of seven days. The steroids,[2] dissolved in 0.2 cc. of sesame oil, were administered once a day either by subcutaneous injection or by stomach tube. In each experiment the substances to be compared were administered in equimolar doses. The androgenic and myogenic activity of the steroids was determined according to Hershberger *et al.* (11). In some instances also the weight of the preputial glands was taken as an androgenic index. Potency ratios were calculated according to Bliss (12).

Qualitative tests as well as approximate determinations of methyltestosterone and its n-hexyl enol ether were carried out with a Perkin-Elmer model 21 infrared spectrophotometer.

RESULTS

When administered subcutaneously all tested enol ethers of methyltestosterone showed an activity significantly lower than that of the parent compound (Table 1).

TABLE 1. ANDROGENIC AND MYOGENIC ACTIVITY OF SOME ENOL ETHERS OF METHYLTESTOSTERONE GIVEN BY SUBCUTANEOUS ROUTE

Treatment	Dose/rat/day		Number of animals	Organ weight (mg./100 gm. body weight)*		
	μMoles	mg.		Seminal vesicles	Ventral prostate	Levator ani
Controls	—	—	10	5.8±0.3	15.9±0.8	20.0±1.4
Methyltestosterone	1	0.302	30	45.8±4.0	99.7±4.9	63.4±2.9
Methyltestosterone ethyl enol ether	1	0.330	10	19.8±1.6	70.5±4.8	38.6±2.0
Methyltestosterone chloroethyl enol ether	1	0.364	10	24.3±2.0	88.9±6.5†	44.3±2.8
Methyltestosterone n-butyl enol ether	1	0.358	10	11.8±0.8	59.0±4.0	27.9±1.4
Methyltestosterone iso-butyl enol ether	1	0.358	10	14.4±0.5	61.9±3.9	30.6±1.0
Methyltestosterone sec-butyl enol ether	1	0.358	10	23.8±1.0	88.0±2.7†	40.5±2.8
Methyltestosterone n-hexyl enol ether	1	0.386	10	15.6±0.9	60.0±3.5	30.2±1.2
Methyltestosterone cyclohexyl enol ether	1	0.384	10	19.6±1.0	63.6±2 8	31.4±1.5
Methyltestosterone phenyl enol ether	1	0.378	10	14.0±0.5	60.2±3.0	28.4±1.2
Methyltestosterone benzyl enol ether	1	0.392	10	18.2±1.0	71.3±2.8	34.0±1.3

* Mean values and standard errors are presented.
P values (relative to methyltestosterone-treated group) are <.001 for all the figures, with the exception of those marked by †, whose P is <.025.

When given by oral route, on the contrary, the activity of the assayed enol ethers was constantly and significantly greater than that shown by methyltestosterone (Table 2).

A more precise evaluation of this increase of activity, measured at three dosage levels of each compound, is presented in Table 3. The calculation of the relative potencies of the tabulated enol ethers showed that they are

[2] For physical constants see a previous paper by Ercoli and Gardi (1).

TABLE 2. ANDROGENIC AND MYOGENIC ACTIVITY OF SOME ENOL ETHERS OF METHYLTESTOSTERONE GIVEN ORALLY

Treatment	Dose/rat/day		Number of animals	Organ weight (mg./100 gm. body weight)*		
	μMoles	mg.		Seminal vesicles	Ventral prostate	Levator ani
Controls	—	—	20	5.7±0.2	14.2±0.6	19.7±0.8
Methyltestosterone	1	0.302	40	12.5±0.6	60.3±2.0	29.6±1.0
Methyltestosterone	1.5	0.453	20	15.6±1.2	67.6±3.3	35.0±1.7
Methyltestosterone	2	0.604	50	17.2±0.7	75.5±2.0	37.0±1.0
Methyltestosterone ethyl enol ether	1	0.330	10	17.2±2.6‡	73.3±5.8§	36.3±2.2
Methyltestosterone n-butyl enol ether	1	0.358	10	23.9±1.7	86.2±8.0	42.0±2.9
Methyltestosterone iso-butyl enol ether	1	0.358	10	25.3±2.9	89.7±4.5	39.6±3.0
Methyltestosterone sec-butyl enol ether	1	0.358	10	22.6±2.3	90.6±9.6	39.6±1.8
Methyltestosterone n-amyl enol ether	1	0.372	10	41.4±3.4	103.9±4.9	53.0±3.0
Methyltestosterone iso-amyl enol ether	1	0.372	10	27.2±1.4	80.5±6.2	41.9±2.9
Methyltestosterone n-hexyl enol ether	1	0.386	20	33.9±2.0	94.3±5.4	46.3±1.8
Methyltestosterone (2-methyl)-pentyl enol ether	1	0.386	20	32.9±1.6	85.9±3.8	49.0±1.9
Methyltestosterone (2-ethyl)-butyl enol ether	1	0.386	10	22.5±2.6	80.0±3.4	34.4±1.7¶
Methyltestosterone n-heptyl enol ether	1	0.400	20	33.8±2.0	91.5±5.4	49.4±2.2
Methyltestosterone n-octyl enol ether	1	0.414	10	22.0±2.6	64.7±5.3	37.3±3.2
Methyltestosterone cyclohexyl enol ether	1	0.384	20	35.9±2.0	100.3±4.5	49.2±2.2
Methyltestosterone benzyl enol ether	1	0.392	10	21.7±2.6	82.6±5.5#	40.0±2.3†

* Mean values and standard errors are presented.
P values (relative to 1 μMole methyltestosterone treated group) are indicated as follows: †, <.005; ‡, <.01; §, <.025; ¶, <.05; #, <.40; for all the other figures P<.001.

3.8 to 8.6 times more androgenic and 2.7 to 5.0 times more myogenic than methyltestosterone.

Table 4 presents the results of *in vivo* and *in vitro* experiments directed to ascertain whether or to what extent the enol ethers are hydrolyzed in the gastric medium. After varying time intervals from the administration of the drug less than 1% of Δ^4-3-ketone (calculated from the recovered enol ether) could be isolated from the removed stomach of the rat. Enol ethers of methyltestosterone either dissolved in oil or suspended in water were

TABLE 3. RELATIVE ANDROGENIC AND MYOGENIC ACTIVITIES OF METHYLTESTOSTERONE AND SOME OF ITS ENOL ETHERS GIVEN ORALLY

Treatment	Dose/rat/day		Number of animals	Organ weight (mg./100 gm. body weight)*			
	μMoles	mg.		Preputial glands	Seminal vesicles	Ventral prostate	Levator ani
Controls	—	—	10	30.3±3.0	5.9±0.2	13.5±1.0	18.8±0.7
M.T.	2	0.604	10	49.8±4.0	13.5±1.0	55.5±2.9	34.3±2.0
M.T.	4	1.208	10	50.0±5.8 (1)	19.5±1.2 (1)	66.8±3.7 (1)	41.3±2.0 (1)
M.T.	8	2.416	10	70.7±5.0	24.6±0.9	72.5±4.1	49.2±2.3
M.T.n-5	0.5	0.186	10	53.7±4.1	13.0±1.0	56.7±4.1	30.1±3.7
M.T.n-5	1	0.372	10	57.9±5.5 (5.3)	17.4±1.8 (3.8)	66.4±5.1 (4.5)	35.6±2.5 (2.7)
M.T.n-5	2	0.744	10	70.4±5.1	26.0±1.9	76.6±3.3	46.6±3.1
M.T.c-5	0.5	0.185	10	61.1±3.0	15.2±0.8	60.4±6.0	36.2±2.3
M.T.c-5	1	0.370	10	62.8±7.4 (8.6)	24.6±1.2 (6.3)	77.4±4.0 (7.8)	42.5±0.9 (4.3)
M.T.c-5	2	0.740	10	76.2±7.6	31.3±2.1	92.4±3.3	48.6±1.6
M.T.n-6	0.5	0.193	10	55.6±3.0	13.7±0.8	57.8±3.5	34.9±2.2
M.T.n-6	1	0.386	10	60.9±4.5 (5.7)	18.6±1.4 (4.5)	61.1±4.5 (4.6)	36.2±2.1 (3.7)
M.T.n-6	2	0.772	10	66.2±6.9	29.0±1.8	82.5±5.0	50.9±2.7
M.T.c-6	0.5	0.192	10	57.3±2.3	13.8±1.1	54.5±3.3	34.8±1.2
M.T.c-6	1	0.384	10	60.0±5.6 (7.2)	21.3±1.5 (5.7)	78.6±5.6 (7.2)	39.9±2.1 (5.0)
M.T.c-6	2	0.768	10	84.4±5.6	35.2±1.6	103.2±2.2	60.4±2.5
M.T.iso-6	0.5	0.193	10	57.3±2.9	14.6±1.1	61.5±2.9	37.0±1.1
M.T.iso-6	1	0.386	10	63.7±3.0 (6.9)	23.9±2.2 (6.3)	71.2±3.7 (6.9)	38.2±1.9 (4.1)
M.T.iso-6	2	0.772	10	68.5±5.8	34.1±2.9	87.7±5.1	50.5±3.3

* Mean values and standard errors are presented. Numbers in parentheses indicate the relative potencies of each compound to methyltestosterone (=1).
Steroid designations: M.T., methyltestosterone; M.T.n-5, methyltestosterone n-amyl enol ether; M.T.c-5, methyltestosterone cyclopentyl enol ether; M.T.n-6, methyltestosterone n-hexyl enol ether; M.T.c-6, methyltestosterone cyclohexyl enol ether; M.T.iso-6 methyltestosterone (4-methyl)-pentyl enol ether.

TABLE 4. RECOVERY OF METHYLTESTOSTERONE AFTER INCUBATION OF METHYLTESTOSTERONE N-HEXYL ENOL ETHER IN ACIDIC MEDIUM

Incubation in	Per cent of methyltestosterone found after		
	15′	30′	60′
Glycine-HCl buffer at pH 2.5	<1	3	18†
	<1	<1	1, 5‡
Human gastric juice	<1	3	10†
Rat stomach	<1	<1	<1§

† Experiment performed adding 100 mg. of finely powdered compound to 5 cc. of acidic medium, maintained under stirring at 37° C.

‡ Experiment performed adding 100 mg. of compound dissolved in 2 cc. of sesame oil to 5 cc. of buffer, maintained under stirring at 37° C.

§ Experiment performed administering to each rat 2 mg. of compound in suspending vehicle. Figures are per cent values of the amount of enol ether recovered from pooled gastric washing of 3 animals.

incubated with glycine hydrochloride buffer (pH 2.5) or with human gastric juice. No appreciable change of the enol ethers could be detected after 15, 30 and 60 minutes.

DISCUSSION

Previous papers (6, 7) from this laboratory have shown that the enol ethers of androstenedione, of testosterone and its esters, assayed by subcutaneous route, exert a very weak androgenic action when compared with that of the corresponding parent steroids. On the contrary the present experiments show that only partial loss of activity occurs in the enolic derivatives of methyltestosterone given by subcutaneous route.

The most significant result of this investigation is that the assayed enol ethers of methyltestosterone are far more active than the parent ketone if they are administered orally. The degree of activity of these compounds depends on the nature of enol ether-forming alcohols. If the carbon chain in the alcohol consists of 5 to 7 carbon atoms (in a straight or cyclic chain) optimal effectiveness is apparently reached. In branched chains the longest chain determines the activity rate (see in Table 2 the activities of (2-ethyl)-butyl and (2-methyl)-pentyl ethers of methyltestosterone).

Because of the easy hydrolysis of enol ethers in the presence of acids one would expect that parent ketones are regenerated through the gastric medium of the rat. The data in Table 4, however, show that such hydrolysis takes place only to a limited extent. We do not know yet whether the steroid is absorbed as an enol ether or any other form. From our findings, however, it becomes obvious that the metabolism of enol ethers is different from that of the parent steroids and also varies according to the route of administration.

Further investigations may provide the explanation for the paradoxical behavior of a number of enol ethers (not only of methyltestosterone but of

many other androgenic, progestational and corticoid Δ^4-3-ketones) being more active than the parent compounds when orally administered and less active by the parenteral route.

It should be noted also that qualitatively the pattern of activity of methyltestosterone is only slightly modified by its conversion to enol ethers (Table 3). In forthcoming papers we shall report striking examples of change in activity brought about by enol etherification of Δ^4-3-keto-steroid hormones.

Acknowledgment

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THE INTERACTION OF ALDOSTERONE AND OF CHOLESTEROL IN THE *in Vitro* BIOSYNTHESIS OF ESTROPROTEIN[1]

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ABSTRACT

Earlier observations on antagonistic behavior between pairs of steroids in the enzyme-catalyzed biosynthesis of their protein-bound forms have been extended to the study of interaction of aldosterone and of cholesterol with estrone-16-C^{14}, as analyzed by methods previously described. It has been demonstrated that aldosterone suppresses, while cholesterol leaves unaffected, hepatically-catalyzed estroprotein biosynthesis *in vitro*. The data suggest that the nature of the inhibition by aldosterone may be competitive, as earlier shown for cortisol in parallel studies from this laboratory. Analysis of the behavior of labeled cholesterol in the system reveals that the liver functions to enhance the association with protein of radioactivity originating from this substrate without being obligatory for the process. Cholesterol does not interfere with simultaneous estroprotein biosynthesis presumably because it does not utilize, selectively, the same protein carrier.

PREVIOUS studies from these laboratories have demonstrated the presence of an enzyme system in rat liver which catalyzes the association of certain steroid hormones or their metabolites with protein of the homologous serum incubation medium (1, 2). Studies with C^{14}-labeled steroids in the system (1–3) have revealed that a significant proportion of the products of incubation of estrone and estradiol (1, 2) as well as of cortisol and corticosterone (3) were so bound to a protein which migrated with the electrophoretic mobility of albumin. It was further demonstrated that there was a competitive interaction between these cortical steroids and the estrogens with respect to association with protein carrier under the conditions described (2–4). Depending on the molar ratios present, estroprotein formation could be inhibited or even suppressed.

It was of interest to extend these studies to the behavior of additional steroidal substances. The interaction of two such compounds with estrone in this system is reported in the present communication. The results indicate that aldosterone inhibits, while cholesterol, chosen as an "inert" control substance, does not affect hepatically-catalyzed estroprotein biosyn-

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thesis *in vitro*. The behavior of labeled cholesterol in the incubation system is also described. A preliminary note based upon a portion of these data has appeared (5).

METHODS

The procedures used in the present study, with exceptions and additions specifically noted below, have been described in detail elsewhere (1–3, 6).

Incubation was carried out with or without surviving rat liver mince in 2 ml. of an homologous serum medium with isotopic steroid added as previously described (1). The gas phase was 95% O_2 – 5% CO_2. Where indicated, unlabeled cholesterol or aldosterone were added in a total volume of 0.05 ml. 95% ethanol. Controls contained the equivalent volume of ethanol. At the end of the 2-hour incubation period, the vessel contents were chilled rapidly, centrifuged in the cold to eliminate tissue residue, and one aliquot of the medium fractionated by a cold acetone procedure. Radioactivity measurements were carried out on the resultant fractions as previously described. Twenty microliters of the serum medium were also removed for electrophoretic resolution in Veronal-acetate buffer at pH 8.6. The dried strips were analyzed for radioactivity in an automatic windowless gas-flow counter at successive half-centimeter intervals. They were then stained for protein with bromphenol blue, and photographed against the curves representing distribution of radioactivity plotted on the same scale. Further details, were required, will be found in the legends to the Figures.

RESULTS AND DISCUSSION

1. *The behavior of cholesterol-4-C^{14}*

Figure 1 reveals the pattern of distribution of radioactivity originating from cholesterol-4-C^{14}[2] incubated in the *absence* of liver tissue. This distribution bears a limited resemblance to the results observed when the parallel incubation was performed in the presence of surviving liver (Fig. 2). It will be noted that while in both cases a considerable proportion of total radioactivity remained at or near the origin, presumably representing unbound sterol (*cf.* also 7), additional radioactivity derived from cholesterol-4-C^{14} was distributed throughout the serum proteins. Similar results were obtained with Δ^4-cholestenone-4-C^{14}[3].

A clear difference may be detected on closer comparison between the control and the sample incubated in the presence of surviving liver. The radioactivity originating from cholesterol incubated with serum alone was found to be distributed in the proteins after electrophoretic separation in such a way that decreasing amounts of isotope were found in successively faster-moving components (Fig. 1). In contrast, incubation in the presence

[2] U. S. Nuclear Corp. In view of the unusual sensitivity of this compound to irradiation-induced decomposition, precautions were taken to minimize this effect by shortest possible storage time in ethanol solution at refrigerator temperature, and to establish its purity and homogeneity prior to use. The sample gave a single peak of radioactivity following reversed-phase paper chromatographic analysis by a modification of the method of Kodicek and Ashby (24). The developing solvents were methanol: 0.001 N HCl (90:10 v/v).

[3] Tracerlab, Inc.

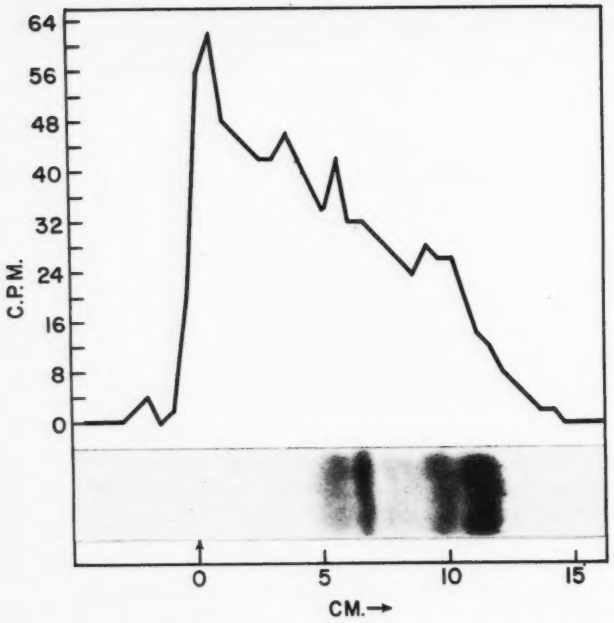

FIG. 1. Distribution of radioactivity after the incubation of cholesterol-4-C^{14}, 0.005 μM/ml. rat serum, in the absence of tissue. Twenty μl. of the resultant medium were subjected to paper electrophoresis by the horizontal open strip method, using Whatman No. 1 paper strips, 1.5 inches wide. Electrophoresis was carried out in barbiturate buffer (Veronal, 0.038 M; sodium acetate, 0.029 M; sodium chloride, 0.011 M; $\mu = 0.08$; pH adjusted to 8.6) at a constant current of 0.5 ma./cm. for 16 hrs. For subsequent analysis, see text.

of liver tissue under similar conditions resulted in a concentration of such radioactivity in the intermediate globulin region consisting principally of α_2- and β-globulins (Fig. 2). In either case the albumin area was very low in isotope concentration. Although the nature of the radioactive substances present in association with the globulins after incubation is not known, it would appear likely that this phenomenon represents sterol-lipoprotein formation, possibly to specific proteins contributed by the liver, and quantitatively enhanced by the presence of this tissue in the medium. Notwithstanding its relatively small magnitude, the enhancement was consistent and statistically significant. In 16 samples of serum incubated with cholesterol-4-C^{14} in the presence of surviving liver, $55.2 \pm 1.7\%$[4] of the total radioactivity present was associated with protein components as determined by the analytical methods described; the corresponding value for 9 control samples was $46.0 \pm 2.8\%$.[4] The P-value of the difference was <0.01.

[4] Mean and S.E.M.

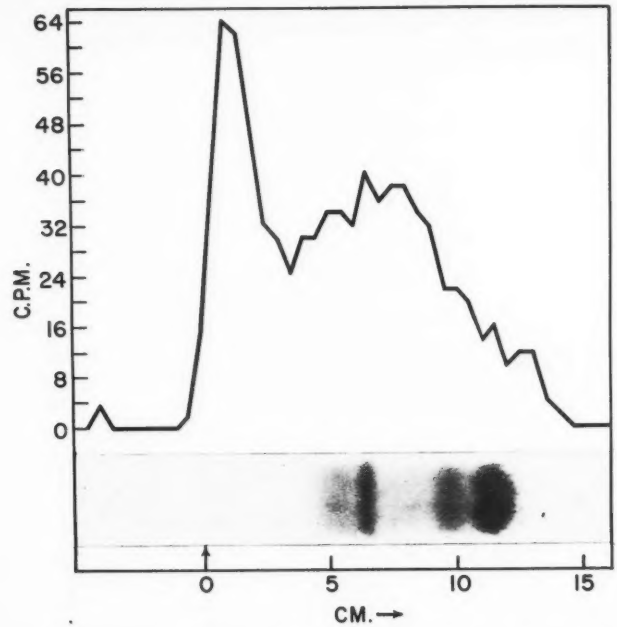

FIG. 2. The distribution of radioactivity after the incubation of cholesterol-4-C^{14}, 0.005 μM/ml. rat serum, in the presence of 250 mg. rat liver mince per total 2 ml. incubation medium. Analytical conditions as in Figure 1 and text.

Oxidation products from the cholesterol substrate were undoubtedly formed in the presence of surviving liver, as has frequently been demonstrated (8–11). Whether the differences in C^{14}-distribution were attributable to these metabolites is unknown. A similar phenomenon of oxidation may have occurred in the controls (*cf.* Bergström and Wintersteiner, 12).

Cholesterol-4-C^{14} subjected to paper electrophoretic analysis in buffer without protein did not migrate significantly from the origin, nor was there any radioactivity in the region corresponding to the mobility of any of the serum proteins, had they been present.

It may be concluded, therefore, that in contrast to the requirement for hepatic intervention in the formation of protein complexes with the estrogens (1, 2; *cf.* also below), with cortisol and corticosterone (3), and with testosterone,[3] 21-desoxycortisol[5] and Δ^4-androsten-11 β-ol, 3, 17-dione[5] (13) the liver is not *obligatory* for the process involving cholesterol or Δ^4-cholestenone,[3] at least at the low levels of concentration herein reported. As indicated above, however, the association of radioactivity

[5] The following compounds used in these studies were generously provided by the Endocrinology Study Section, U. S. Public Health Service: *d,l*-aldosterone-21-monoacetate, 21-desoxycortisol-4-C^{14}, and Δ^4-androsten-11β-ol, 3,17-dione-4-C^{14}.

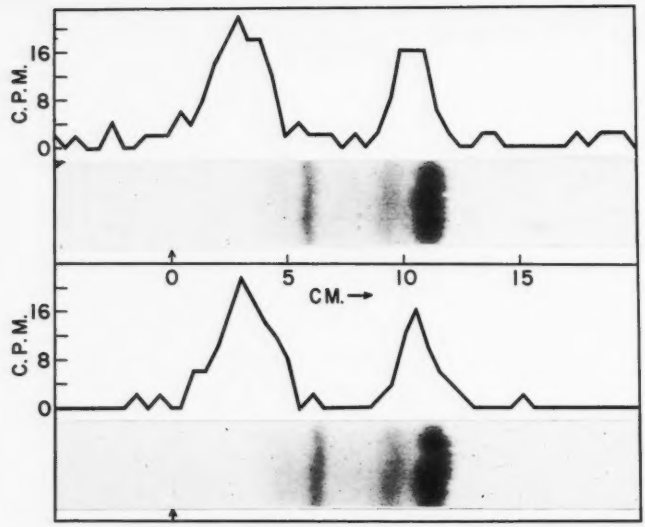

FIG. 3. The failure of cholesterol to exert an inhibitory influence on the liver-catalyzed binding to albumin to radioactivity originating from estrone-16-C^{14}. The upper half of the Figure represents the control sample (estrone-16-C^{14}, 0.032 μM/ml.; the 2 ml. total serum medium also contained 250 mg. minced rat liver tissue and 0.05 ml. 95% ethanol). The lower half of the Figure represents the results of a parallel incubation carried out under identical conditions with the exception that the 0.05 ml. 95% ethanol added per 2 ml. total incubation medium contained unlabeled cholesterol to a final concentration of 0.647 μM/ml. Analytical conditions as in Figure 1 and text.

derived from cholesterol-4-C^{14} with the serum proteins of the incubation medium is quantitatively enhanced in presence of liver tissue.

The property of cholesterol in formation of associations with serum proteins has long been recognized (*cf.* 14–16). While under special circumstances and in particular species, notably the human, association of cholesterol and of lipid may occur predominantly with the β-globulin components of the serum, with secondary concentrations in the α_1-fraction (*cf.* 17–19), it appears that there is less selectivity in the association of cholesterol or its metabolites with a particular rat serum protein under the present conditions. Similarly nonspecific distribution of total lipid in rat serum was observed by Morris and Courtice (20) and by Hillyard *et al.* (21).

2. *The failure of cholesterol to influence liver-catalyzed estroprotein biosynthesis*

Figure 3 depicts the failure of cholesterol to exert any significant influence on the process of estroprotein formation. Shown at the top portion of the Figure is the estrone-16-C^{14} control sample; at the bottom, estrone in

the presence of unlabeled cholesterol. The first peak in each instance represents unbound radioactivity; the second reflects hepatic intervention in promoting the association of radioactivity originating from estrone-16-C^{14} with the serum proteins, notably albumin (2). It may be observed that the area of radioactivity defined by the second peak is similar in both instances. Thus, cholesterol, although it is rendered protein-bound in the system (Figs. 1, 2), does not interfere with simultaneous estroprotein biosynthesis, presumably because it does not utilize, selectively, the same protein carrier.

3. *The inhibitory effect of aldosterone on liver-catalyzed estroprotein biosynthesis*

In striking contrast to the failure of cholesterol to influence the course of estroprotein formation *in vitro*, it may be observed in Figure 4 that aldosterone,[5] in progressively increasing concentrations from zero in the left-hand third of the Figure, produces conspicuous inhibition of protein complex formation with labeled estrone or its metabolites, as seen by the graded decline in the second peak of radioactivity. The absolute control for this series of experiments is shown in Figure 5, which depicts the results of estrone-16-C^{14} incubation in the absence of surviving liver tissue and aldosterone. In confirmation of previous studies with labeled estrogens,

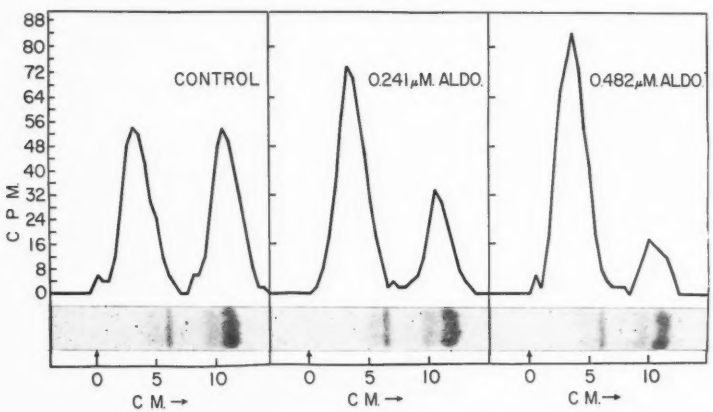

FIG. 4. The inhibitory influence of aldosterone on the liver-catalyzed binding to albumin of radioactivity originating from estrone-16-C^{14}. All samples contained 250 mg. rat liver mince in a total of 2 ml. serum incubation medium. The estrone-16-C^{14} concentration was constant at 0.098 μM/ml. In the middle and right-hand samples, *d,l*-aldosterone-21-monoacetate in 95% ethanol was added in the concentrations shown per ml. The latter were calculated in this and in Figure 6 on the basis that $\frac{1}{2}$ of the *d,l*-mixture is biologically effective (*cf.* 23). The control (left-hand) sample contained the compensatory 0.05 ml. 95% ethanol vehicle per 2 ml. total incubation medium. Analytical conditions as in Figure 1 and text.

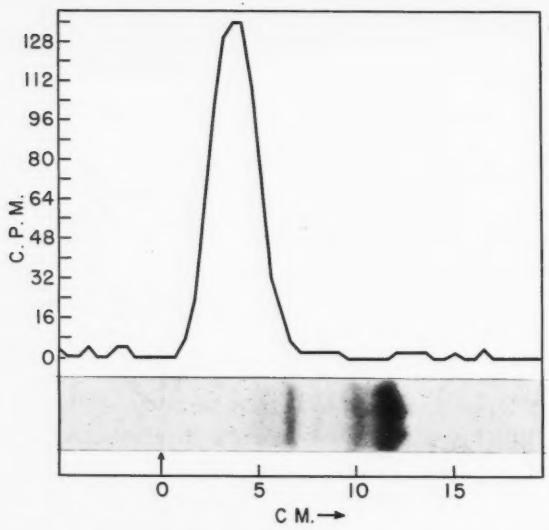

FIG. 5. Liverless incubation control for experiment depicted in Figure 4, containing the estrone-16-C^{14} concentration indicated in the latter, but no aldosterone or liver tissue. Note failure of association of radioactivity with protein in the absence of surviving liver, as has earlier been described (*cf.* 1–3).

cortisol, and corticosterone, hepatic intervention is obligatory for significant amounts of protein binding (1–3). These data are in further contrast to the absence of a similar requirement in the case of cholesterol or of Δ^4-cholestenone under these conditions (*cf.* above).

The mechanism of the inhibition by aldosterone of estroprotein biosynthesis in the present studies is obscure. Analysis of the results by the series

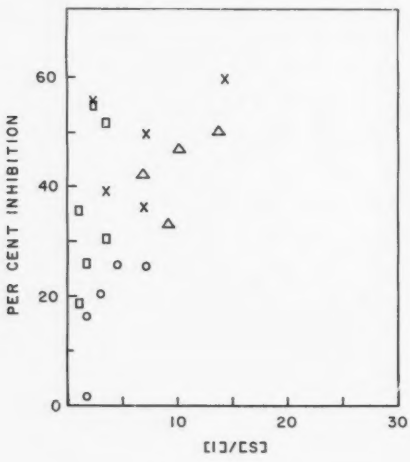

FIG. 6. Pattern of inhibition by aldosterone of estroprotein biosynthesis *in vitro*. Incubation and analytical methods as in text and Figure 1, respectively. Ordinate, per cent inhibition (based on change from control without added aldosterone), of binding of radioactivity originating from estrone-16-C^{14} substrate, (S), in the presence of varying amounts of *d,l*-aldosterone-21-monoacetate inhibitor, (I), as determined from proportion of total radioactivity associated with albumin area of electrophoretic strip. Abscissa, molar ratios of inhibitor: substrate. The range of substrate concentrations was from 0.06 to 0.098 μM/ml. Each type of symbol represents the range of values obtained with a single experiment, of which 4 are shown.

of conventional methods used to identify competitive antagonism does not clearly indicate, as it did with earlier findings on cortisol and estrone (3), that the inhibition is competitive. It is likely, however, as discussed earlier (3), that the present system is too crude for such rigorous analysis. Figure 6 demonstrates, nevertheless, that the per cent inhibition is in general dependent upon the molar ratio between inhibitor, [I], and substrate, [S], being greater at high, than at low [I]/[S] ratios. The correlation coefficient calculated from these data is 0.508 with a Fisher "t" of 2.738 and a P-value of approximately 0.01. The general pattern of inhibition revealed in Figure 6 appeared also when resolution of the samples was carried out by cold acetone precipitation of the proteins of the incubation medium and their subsequent fractionation as earlier described (1, 3, 6).

The significance of the inhibitory interaction between aldosterone and estrone, as revealed by the present studies (and of cortisol, corticosterone and desoxycorticosterone as described earlier) is unknown. That it is not a general property of steroids is indicated by the negative results with cholesterol. In the case of corticosterone and cortisol, but probably not desoxycorticosterone, it may be a reflection of at least one aspect of their demonstrated suppressive interaction with estrogens in the uterine growth response *in vivo* [*cf.*, however, (22)]. The amounts of aldosterone thus far available have not permitted similar *in vivo* studies to be performed.

Acknowledgment

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THE EFFECT OF GROWTH AND THYROID HORMONES UPON THE REMOVAL BY LAVAGE OF CALCIUM AND STRONTIUM FROM THE SKELETON OF THE RAT[1]

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ABSTRACT

Intraperitoneal lavage was used to remove some of the previously deposited radiostrontium and radiocalcium from the skeletons of rats. The efficiency of radioisotope removal was taken as an index of the incorporation of the radionuclide from the labile into the less-labile portions of bone. The data showed that removal efficiency decreased with time from dosing until the start of lavage. In hypophysectomized rats as compared to normals, removal efficiency was increased at 24 hours after lavage but not at 8 or 48 hours. Supplying growth hormone to hypophysectomized rats decreased the amount of radioisotope that could be removed by lavage at 24 hours after dosing. Thiouracil, when fed to intact rats, increased the amount of radioisotopes that could be removed; thiouracil fed to hypophysectomized rats receiving growth hormone, however, did not produce this response.

ALTHOUGH the kidney and the intestinal tract possess mechanisms that select against strontium-90 in favor of calcium (1), an appreciable amount of dietary radiostrontium is accumulated in bone. The prevention of radiostrontium uptake by bone and/or devising of methods of removal of this nuclide are basic problems in health physics, and their importance necessitates the collection of information about the many factors that influence the deposition of alkaline earth metals in bone.

It has been observed that animals deprived of the pituitary gland do not grow and do not develop a normal skeleton (2). This suggested that some of the hormones of the pituitary may be involved in control of bone formation. Indeed, work has shown that growth hormone may control protein matrix formation in bone (3). Thyroxine has been demonstrated to enhance the protein anabolism fostered by the anterior pituitary hormone (3); thyroidectomy in itself results in a depression of skeletal maturation resembling hypophysectomy (2). These hormones apparently are involved in the control of the normal sequence of events in bone; therefore, it was reasoned that, in the absence of one or both of these hormones, recently-administered radiostrontium and radiocalcium would not become deeply

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incorporated into bone mineral and would be more available for removal. The present study was undertaken to test this hypothesis and gain further insight into the metabolic events in bone.

METHODS

The lavage technique of Talmage *et al.* (4) was chosen as the experimental approach since it could provide information on the mobilization of calcium and strontium and reflect to some degree the state of calcium in bone.

In these studies young male hypophysectomized rats of about 6 weeks of age were obtained from the Charles River Laboratory and were maintained on laboratory rations before being used for experiment. Since removal of the pituitary results in a retardation of growth and maturation, it was felt necessary to match the hypophysectomized animals with intact rats of comparable body size (about 100 gm.) rather than to use animals of equal chronological age. The rats were injected intraperitoneally with 0.5 ml. of a solution containing 50 μc Ca^{45} and 5 μc Sr^{85} (both essentially carrier-free) and then placed on a low calcium diet (cornmeal) until the lavage began. At various time intervals after injection, the rats were anesthetized with sodium pentobarbitol and a cannula was inserted through the inguinal canal or the abdominal wall into the peritoneal cavity. After an initial rinse, 10 ml. of a warm solution containing 0.9% NaCl and 1.0% glucose were administered through the cannula and allowed to equilibrate with the body fluids for one-half hour. The peritoneal lavage solution was then removed, measured, and replaced with 10 ml. of fresh solution. The washings for 3 half-hour periods were combined to reduce the number of analyses required. The lavage experiments lasted from 4 to 6 hours.

Permanganate titration was used to determine stable calcium and an oxalate precipitation method to determine Ca^{45} (5); Sr^{85} was counted directly by means of a well-type scintillation detector. The radioisotope data are expressed as per cent of the skeletal Ca^{45} or Sr^{85} removed by the lavage procedure.

RESULTS

Experiment A, Table 1, presents data that compare alkaline earth metabolism in the hypophysectomized rats to intact rats of equal body weight at 8, 24, and 48 hours after the animals were dosed. It is evident that the amount of removable radionuclide decreased markedly with time from dosing until the start of lavage. This was consistent with the progressive incorporation of the radioisotopes into a less available phase of bone mineral. The results of this experiment verified the observations of Talmage *et al.* (6) that more Sr^{85} was removed than Ca^{45} and that the Sr^*/Ca^* ratio tended to remain substantially uniform during the removal process. Only at 24 hours was it possible to remove more Sr^{85} and Ca^{45} from the skeleton of the hypophysectomized animals than from the controls ($P < 0.05$). Since the amount of stable calcium in the wash solutions was independent of time after dosing, the specific activity of the removed calcium decreased with time after dosing and was always greater for the hypophysectomized rats.

It was then of interest to see whether growth hormone supplied to the hypophysectomized rat could change the response to that resembling a normal animal. For this purpose, a group of hypophysectomized rats re-

TABLE 1. THE REMOVAL OF STRONTIUM 85, CALCIUM 45, AND STABLE CALCIUM FROM THE SKELETONS OF RATS USING INTRAPERITONEAL LAVAGE AT STATED TIME INTERVALS AFTER THE INJECTION OF THE RADIOISOTOPES

Treatment	Hours after Dosing	No. of Rats	Sr^{85}	Ca^{45}	Ca^{40}
			% of dose	% of dose	mg.
Experiment A					
Hypox[a]	8	4	5.3±0.2[b]	4.1±0.2	4.4±0.6
Intact		3	5.0±1.2	4.0±0.5	5.7±0.4
Hypox	24	6	4.0±0.6	3.1±0.3	4.1±0.2
Intact		5	2.1±0.1	1.8±0.1	4.8±0.2
Hypox	48	3	1.5±0.3	0.9±0.1	3.7±0.2
Intact		4	1.2±0.1	0.7±0.1	5.0±0.1
Experiment B					
Hypox	8	2	7.8±1.0	4.9±0.5	1.6±0.2
Hypox+G.H.[c]		4	5.1±0.9	3.1±0.5	2.5±0.3
Hypox	24	4	2.4±0.6	2.4±0.6	2.6±0.3
Hypox+G.H.		2	1.6±0.1	1.3±0.02	3.6±0.3
Hypox	48	3	3.6±0.1	2.5±0.2	—
Hypox+G.H.		4	2.5±0.2	1.4±0.1	—
Experiment C					
Intact+Thiouracil[d]	24	7	4.1±0.3	3.0±0.4	4.0±0.4
Intact		6	3.0±0.1	1.9±0.1	5.6±0.8
Experiment D					
Hypox	24	4	2.8±0.7	2.1±0.7	1.5±0.1
Hypox+G.H.[e]+ Thiouracil		3	1.9±0.3	1.4±0.2	1.6±0.1
Hypox+G.H.[f]		3	1.6±0.5	1.0±0.3	1.8±0.2

[a] Hypophysectomized rats.
[b] Mean ±standard error of the mean.

$$s\bar{x} = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n(n-1)}}$$

[c] For 9 days prior to lavage received a daily subcutaneous injection of 1 mg. of growth hormone (Somar).
[d] Fed a diet containing 0.5% thiouracil for a period of two weeks before lavage.
[e] Fed the 0.5% thiouracil diet as well as receiving daily injections of growth hormone.
[f] Received daily injections of growth hormones as did the rats of Experiment B.

ceived daily subcutaneous injections of 1 mg. each of growth hormone (Somar) for a period of 9 days while the control rats were given isotonic saline. The growth hormone allowed a resumption of growth while the controls, as expected, remained static in weight. The results of Experiment B show that growth hormone produced a metabolic response in the skeleton, when compared to the untreated hypophysectomized rats, that closely resembled that of the intact rats of Experiment A.

Since thyroxine has been shown to increase the response of hypophysectomized rats to growth hormone, it was of interest to investigate its relationship to growth hormone in the calcification process. For this purpose,

normal male rats were pair-fed for 2 weeks on a diet that did or did not contain 0.5% thiouracil. The data of Experiment C show that the thyroid block from thiouracil feeding caused a response in intact rats that was strikingly similar to hypophysectomized rats. It was observed that the removal of Sr^{85} and Ca^{45} was increased and the removal of stable calcium was decreased in the thiouracil-fed animals in comparison to the controls.

These data suggested that stimulation of the thyroid of the hypophysectomized rat might be involved in the response to growth hormone. To test this premise more rigorously, the following experiment was undertaken. Three groups of hypophysectomized rats were formed and fed a normal calcium diet. One group received daily subcutaneous injections of isotonic saline; the second received 1 mg. of growth hormone subcutaneously each day, while the third group, in addition to the growth hormone injections, received 0.5% thiouracil in the diet. From the results (Experiment D), it is evident that growth hormone produced the expected response and that this response was not significantly altered by the presence of thiouracil in the diet. This indicates that the pituitary hormone does not necessarily act on the bone mineralization process by first stimulating the thyroid gland.

DISCUSSION

In the peritoneal lavage studies of Talmage *et al.* (4), parathyroidectomy had no effect on removal of radiocalcium or radiostrontium injected 24 to 48 hours prior to the start of lavage; however, parathyroidectomy did reduce the removal of radioactivity injected 2 to 3 weeks prior to the lavage. These experiments provided additional evidence that calcium is removed from bone by two processes. One process, as deduced from the results of lavage begun soon after dosing with radioisotopes, is an equilibration between the fluid media of the body and the labile portions of bone. The second, as indicated by the longer-term studies, is removal from deeper areas of bone.

The study of Talmage *et al.* (4) is pertinent since the results we are reporting were obtained from experiments in which intraperitoneal lavage was always begun within 48 hours after injection of the radioisotopes. Therefore, the radioactivity in the washings reflected the radioactivity of the labile portions of bone rather than that of the deeper areas of bone. The data of Experiment A show that a progressive incorporation of radioisotopes into the less-labile portions of the skeleton occurred for both groups. The similarity of the values between groups at 8 and 48 hours after the injection of radioactivity is in sharp contrast to the 1.7 times as much Ca^{4} and Sr^{85} that could be removed at 24 hours from the skeletons of the hypophysectomized rats as from the skeletons of intact rats. This is taken a an indication that a deficiency of growth hormone resulted in a decreas in the rate of incorporation of the alkaline earth metals from the labile int

the less-labile portions of bone. The data of Experiment B confirm that growth hormone participates in altering this rate of incorporation.

The decreased removal of stable calcium seen in the hypophysectomized rats suggests that growth hormone also influences the resorption of calcium from the relatively deeper portions of bone.

The thiouracil studies show that depression of thyroid hormone production could produce a condition similar to hypophysectomy in that stable calcium in the washings was reduced while the amounts of radionuclides were increased. The mechanism of action of growth hormone was not that of stimulating the thyroid, however, since it was shown that administration of growth hormone produced the same effect when given to hypophysectomized rats or hypophysectomized rats fed thiouracil.

The exact mechanism of action of growth hormone and thyroid hormones, with regard to calcification, is unknown. These hormones have in common the alteration of the overall cellular metabolism of the body, though they do not necessarily operate via the same mechanisms. The present observations on bone may be the result of general effects rather than specific skeletal effects. *In vitro* studies have shown that the incorporation of Ca^{45} and Sr^{85} into the less-labile fraction of bone mineral is under the control of bone cells (7); perhaps thyroid and growth hormones can regulate the level of metabolic activity of these cells to produce the effects seen here. Also, growth hormone may exert its effect by way of its previously recognized action on protein synthesis (3).

In reference to radiostrontium removal, it was noted that hypophysectomy and thiouracil feeding resulted in a 1.6-fold increase in removal of Sr^{85} when compared to the control animals. Since the total amount of Sr^{85} removed was only about 4% of the skeletal content, this was not a significant decrease in the body burden. This re-emphasizes the rapid fixation of radiostrontium into bone and the reason for the general lack of success in removal attempts. Perhaps thiouracil administration, under certain conditions, may increase the effectiveness of removal procedures such as artificial kidney, ion exchange, or citrate therapy.

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MODIFICATION OF DEGENERATIVE JOINT DISEASE OF MICE BY SOMATROPHIN (STH)[1,2]

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ABSTRACT

Male mice of strain C57BL Jax 6 were treated with increasing doses of the bovine growth hormone Somar for 5 months starting at the age of 6 weeks. The observations were extended into old age. The joints of these mice were studied microscopically, and the progress of articular growth, of old age changes and of osteoarthritis was analyzed. As compared with conditions in untreated controls, STH promoted growth and old age changes of the articular cartilage; it also accelerated the onset and increased the incidence and severity of degenerative joint disease. The relationship of spontaneous osteoarthritis occurring in ageing mice to that seen after administration of STH as well as in acromegalic patients is discussed. It is felt that on the basis of microscopic findings a distinction between these two types of arthropathies cannot be made with certainty. A slight transitory thyrotrophic effect but no other trophic effects of Somar were noted.

IN GUINEA pigs and mice, anterior hypophyseal transplants and crude extracts stimulated growth of the epiphyseal cartilage and promoted the catabolic processes of ageing in this tissue. Thus, the hormone accelerated the progress of the skeletal time curve. In the joints, this was reflected by premature evolution and accentuation of osteoarthritis (1, 2). In rats, crude as well as purified anterior hypophyseal extracts likewise produced arthropathies (3–5). In continuation of our previous investigations, the course of articular ageing and its relationship to the development of osteoarthritis was studied in mice given purified STH, and the results obtained are herewith presented.

MATERIAL AND METHODS

Seventy-three male mice of the closely inbred strain C57BL Jax 6 raised in our laboratory were used. The animals were kept on a stock diet of Purina laboratory chow and water ad libitum and divided into two groups. (1) *The control group* comprised 34 un-

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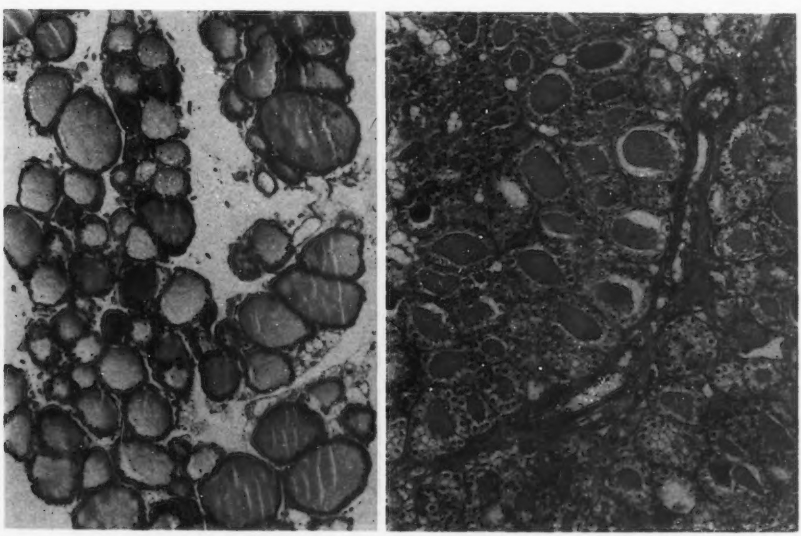
treated mice. (2) *The test group* consisted of 39 mice injected with a solution of the hormone obtained by dissolving 50 mg. of the dry powder in 25 cc. 0.9% saline at a pH of 10.2. From the age of 6 weeks on, the animals received 6 subcutaneous injections daily for 5 consecutive months. The daily doses were increased with advancing age, as follows: 40 μg for the first 10 days, 60 μg for the following 20 days, 80 μg for the next 20 days, 100 μg for another 40 days, and 140 μg for the last 60 days. After 5 months the treatment was discontinued.

Weights were taken at regular intervals. The animals were sacrificed at the age of 18 months. This is the age at which the peak incidence of spontaneous osteoarthritis is reached in mice of this strain (2). Some animals died or were killed before that age, and a few others were allowed to live longer. Details are shown in the table. At necropsy, the kneejoints including the upper end of the tibia and the lower end of the femur were removed as a whole, fixed and decalcified in Bouin's solution, and embedded in paraffin. Semiserial sections were stained with hematoxylin and eosin. Thyroids, pancreas, adrenals and thymus were likewise saved for microscopic examination. The findings in the joints of the test animals were compared with those of the controls, and the data were analyzed statistically with the Chi square method.

OBSERVATIONS

Weights. The weights of the mice treated with STH did not differ from those found in the controls, not even during the period of active growth.

Endocrine Organs. Spot checks were made in animals killed during the period of injecting. In these mice, the thyroids were slightly enlarged and congested. There was hypertrophy of the follicular epithelium which was



Sections through the thyroid of 3-month old mice of strain C57BL Jax6. ×140.

FIG. 1 (left). Untreated mouse. Resting thyroid.

FIG. 2 (right). Mouse treated with STH. Hypertrophy of the follicular epithelium with softening of colloid.

of tall columnar instead of cuboidal type usually seen in mice of this strain. While in the controls the colloid stained deeply with eosin and was homogeneous, it had under the influence of STH become pale and soft, and showed scalloped edges (Figs. 1, 2). After discontinuation of the treatment, the thyroids returned to a resting state. The findings thus resembled those observed in animals treated with crude bovine anterior hypophyseal extract (1). However, the changes produced by STH were less accentuated than those seen after injections of the crude extracts.

Pancreas, adrenals and thymus of the injected animals were not remarkable.

Joints. According to a classification established previously in detail (1), articular old age changes characterized by hyperplasia, hypertrophy and degeneration of cartilage can be distinguished from osteoarthritis, as long as the joint surface remains intact. Osteoarthritis is either of predominantly hypertrophic or ulcerative type with marginal outgrowths and deformities of the joint cavity. Superficial erosions were considered as slight lesions, large and deep ulcerations with considerable hypertrophic cartilaginous or osseous outgrowths and deformities of the joints were considered as severe lesions. Figures 3–8 demonstrate some of the representative findings.

The results of the microscopic examination are summarized in the table.

TABLE SHOWING THE FINDINGS IN THE KNEEJOINTS OF UNTREATED MICE AND OF MICE TREATED WITH GROWTH HORMONE

Series	No. of mice	Mean age months (range)	Articular findings						
			No change		Age changes		Osteoarthritis		
							Total		Severe
			No.	%	No.	%	No.	%	No.
Untreated Controls	34	18.6 (12–27)	3	9	20	59	11	32	4
Growth Hormone	39	17.7 (12–20)	0	0	9	23	30*	77	26**

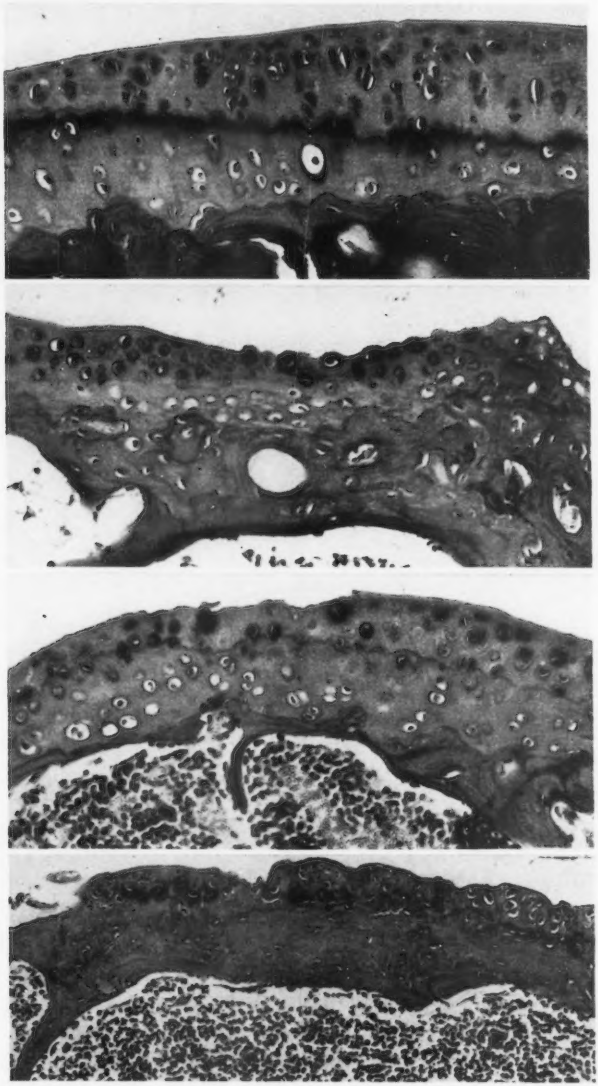
* Untreated: Growth Hormone $P < .001$.
** Untreated: Growth Hormone $P < .01$.

RESULTS

(*1*) *Unchanged Joints.* Of 34 untreated mice 3 (9%) had unaltered joints, while there were no unchanged joints in the test animals.

(*2*) *Old Age Changes.* In 20 of 34 control mice (59%) articular old age changes were noted. A similar involvement was observed in 9 (23%) of 39 hormone-treated animals.

(*3*) *Osteoarthritis.* Of 34 controls 11 (32%) had osteoarthritis, while of the 39 test animals the joints of 30 (77%) were thus affected. This difference is highly significant statistically—$P < .001$. Moreover, while in the



Sections through the articular surface of male mice of strain C57BL Jax6, 18 months of age. ×200.

FIG. 3 (top). Tibial aspect of the kneejoint of an untreated mouse. No change.

FIG. 4 (second). Metatarsal joint of a mouse treated with STH. Old age change. Hypertrophy and degeneration of cartilage; surface intact.

FIG. 5 (third). Tibial aspect of the kneejoint of a mouse treated with STH. Osteoarthritis slight. Degeneration and hypertrophy of cartilage; shallow erosion of surface.

FIG. 6 (bottom). Tibial aspect of the kneejoint of a mouse treated with STH. Osteoarthritis advanced. Cartilaginous outgrowth with ulceration.

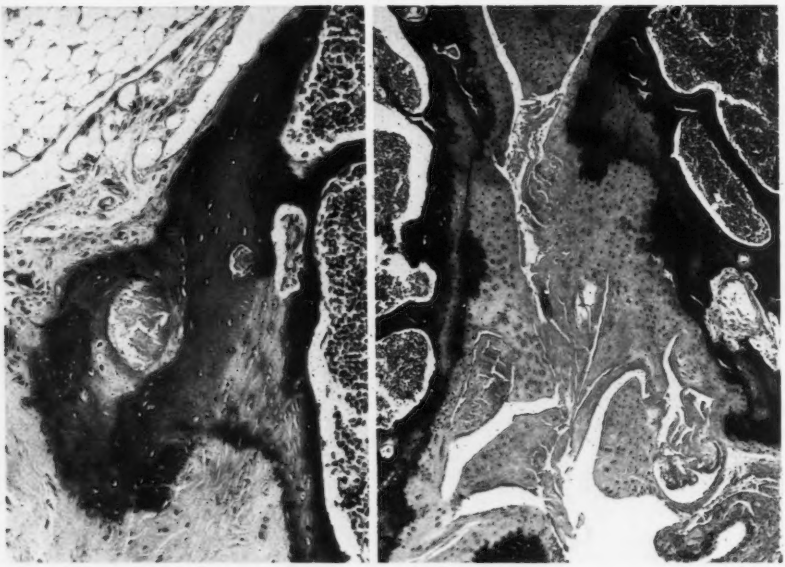


FIG. 7 (left). Tibial aspect of the kneejoint of a mouse treated with STH. Osteoarthritis advanced. Marginal lipping.

FIG. 8 (right). Kneejoint; femur in right, tibia in left part of the figure. Osteoarthritis advanced. Deformity of joint with obliteration of the joint cavity.

controls only 4 (36%) of the articular lesions were severe, 26 (87%) of 30 mice of the test series showed advanced osteoarthritis. This difference in the severity of the osteoarthritic lesions is statistically significant at the 1% level.

DISCUSSION

In male mice of the closely inbred strain C57BL Jax 6 receiving the bovine growth hormone Somar from the age of 6 weeks on for a period of 5 months, both incidence and severity of osteoarthritis were significantly increased as compared to conditions seen in untreated animals. Old age changes were numerically decreased in mice injected with STH. However, this finding does not indicate a slowdown of the ageing process; it actually reflects an accelerated transformation of age changes into arthritic lesions. During the period of injections a slight transitory thyrotrophic effect of STH was observed.

The articular response of mice to the administration of purified STH thus closely resembled that seen after grafting of anterior hypophyses from closely related donor animals or that noted in guinea pigs, rats and mice after injections of crude or acid anterior hypophyseal extracts (1–5). Under all these conditions, STH asserted itself, and the findings were comparable to those seen in acromegaly. The joint lesions found in acromegalic pa

tients are considered to be morphologically specific, and the term acromegalic arthropathy was coined in order to designate a disease entity apart from other forms of non-inflammatory joint diseases, especially from osteoarthritis (1, 5–8). This distinction is essentially based on the view that osteoarthritis is characterized by primary deterioration of the articular cartilage and secondary reactive growth from the part of the articular tissues. In acromegaly, on the other hand, the primary disturbance is thought to consist of a primary reactivation of the dormant growth potential of the cartilage which quickly outgrows its nutritional supply and degenerates. The growth of cartilage in acromegaly would thus not be a reaction to injury as in the case of osteoarthritis but an independent articular response to the excessively produced STH. Certain microscopic criteria, especially the demonstration of increased growth of the middle layer of the articular cartilage or the presence of cytoplasmic fat were considered to prove the specificity of acromegalic arthropathy (5, 8, 9). However, on the basis of some clinical studies and of experiments in rodents it appears increasingly difficult to establish definite criteria that would allow a distinction between the spontaneous arthropathy of advancing age and that due to the effect of STH (2, 10–12). In acromegalic joints as well as in degenerative joint disease, the same type of tissue growth and degeneration, and the same sequence of events were found: Early spontaneous growth of cartilage could easily be detected in joints of untreated ageing mice or ageing humans. Changes in the cartilaginous matrix on the other hand with regression of cells may be observed also during early stages of treatment with STH (1b, 2). The differences that do exist between the two types of joint disease are relative rather than absolute, and seem to lie essentially in a time factor and possibly in different intensities of growth and regressive changes of cartilage. Besides, acromegalic arthropathy does not follow a fixed pattern but the changes show a spectrum of variability according to the age of the patient at the onset of the disease: The younger the individual, the more accentuated the growth response (1b, 2, 8).

In spite of the transitory thyrotrophic effect of Somar it is highly improbable that this contamination should have played a role in the results obtained. Prolonged administration of thyroid hormone accelerated development and differentiation of cartilage but attenuated the evolution of osteoarthritis in mice. The latter effect was attributed to a condensation of the matrix which in turn might inhibit both growth and regression of cartilage (1b). If the transitory stimulation of the thyroid observed in the present experiments had any articular effects at all, it would have counteracted those of STH. However, mice of strain DBA "thyroidectomized" with I^{131} develop a peculiar joint disease which might result from the absence of thyroid secretions as such or possibly from an excess of thyrotrophin. Morphologically, these lesions differ from those of spontaneous osteo-

arthritis; furthermore, mice of strain C57BL Jax 6 such as used in the present investigation were remarkably resistant to this type of joint disease (1c). Therefore, contaminating thyrotrophin does not seem to be involved in the increase and accentuation of osteoarthritis caused by STH.

Under the influence of STH, both articular growth and regressive changes proceeded faster than without hormonal stimulation. The articular cartilage thus reacts readily to changes in the internal environment, especially in the hormonal equilibrium, a fact that is being increasingly recognized. Whether or not the response of the articular cartilage to the administration of STH may be extrapolated to indicate that this hormone is implicated in the pathogenesis of degenerative joint disease in general, is an intriguing question. The results obtained corroborate the view that agents that stimulate growth of cartilage likewise promote the development of osteoarthritis.

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QUANTITATIVE HISTOCHEMICAL DISTRIBUTION OF FREE AND BOUND PANTOTHENATE IN THE RAT ADRENAL AND THE RESPONSE TO ACTH[1,2]

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ABSTRACT

A microbiological assay with *Lactobacillus arabinosis*, previously developed, was used to study the quantitative histological distribution of free and bound (mainly coenzyme A) pantothenate in the rat adrenal. Concentrations were expressed as pantothenic acid per unit protein-nitrogen. Less than 20% of the total pantothenate in microtome sections of the cortex was found to be free, and this smaller fraction was rather uniformly distributed among the cortical zones but it occurred in higher concentration in medulla. ACTH treatment of the rats (25 mg./kg. injected subcutaneously 3 hours before sacrifice) reduced the concentration of free pantothenate in all zones to about the same degree.

Bound pantothenate was evenly distributed throughout the gland, and the ACTH treatment elicited a significant rise in concentration only in fasciculata. This elevation was greater than the fall in concentration of free pantothenate, indicating a mobilization of Co A in this zone in addition to direct synthesis from the free form.

QUANTITATIVE histological distribution of free and bound pantothenate in rat adrenal can be studied more advantageously by microbiological than by microchemical methods. The concentrations of both forms can be measured on the same sample, histological definition can be increased by use of smaller samples and the substances can be measured as a function of their actual biological activity. The present study, which exploits these advantages, extends earlier work on coenzyme A (Co A), as measured chemically, in rat adrenal (1, 2) to permit microbiological determination of both free and bound (primarily as Co A, 3) pantothenate in the discrete zones of the gland. Adrenals from control animals, and from those injected subcutaneously with ACTH, were used.

EXPERIMENTAL

Adrenal glans were obtained from male albino rats (Sprague-Dawley) weighing 350–450 gm., approximately 3 months old, purchased from Holtzman Co., Madison, Wis.

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The rats were housed in a constant climate room (25° ±1° C, 30–50% relative humidity) with controlled illumination (lights on 6 A.M., off 6 P.M.) and fed Purina Fox Chow and tap water *ad libitum*. ACTH (25 mg./kg.) was administered subcutaneously to separate animals in 1 ml. of physiological saline solution 3 hours prior to sacrifice. The animals were killed instantly by a single hammer blow on the head at noon ±15 minutes. The left adrenals were quickly removed, frozen with solid carbon dioxide, transferred to chilled plastic vials and allowed to equilibrate at −15° C for 30 minutes in the manner employed earlier (2). The preparation of serial fresh-frozen circular microtome sections (2 mm. diameter, 16 μ thick) followed the procedure already described (4). One section was brushed flat on an albuminized slide for histological examination after toluidine blue staining (5), the next 4 sections were placed in a culture tube for microbiological assay by the method developed for this purpose (6), and the following section was placed in another tube for protein-nitrogen (P_N) analysis (7).

Microbiological Assay

The micromethod employed (6) is an adaptation of the Novelli-Schmetz procedure in which total pantothenate is measured after enzymatic conversion of bound forms to free pantothenate. *Lactobacillus arabinosis* was employed and the growth response after 48 hours was measured tubidimetrically.

Each assay tube was removed individually from the cryostat, the sections were extracted by "buzzing" agitation with 60 μl. of water for 30 seconds, and then the tube was immersed in boiling water for 45 seconds to stop autolytic release of pantothenic acid from CoA. After clarification by centrifugation for 2 minutes at 7500 *g*., three 10 μl. aliquots of extract were assayed for free pantothenate. The remaining 30 μl. were subjected to enzyme hydrolysis for 6 hours at 37° by the addition of 5 μl. of 0.2% purified intestinal alkaline phosphatase, 10 μl. of 10% suspension of Dowex-treated pigeon liver acetone powder in 0.02 M potassium bicarbonate, and 5 μl. of 0.1 M Tris buffer, pH 8.3. After inactivation of the enzyme system by immersing the tubes in boiling water for 45 seconds, the hydrolyzate was appropriately diluted, clarified by centrifugation and three 10 μl. aliquots assayed for total pantothenate. Standard solutions of CoA, purchased from Pabst Laboratories, Milwaukee, Wis., were assayed along with the adrenal samples in each experiment to check enzyme activity.

RESULTS AND DISCUSSION

In studies by the chemical method (1, 2), highest concentrations of Co A were observed in the outer fasciculata and fascicular-reticular border zone, with intermediary values in the reticular-medullary region. Administration of ACTH increased concentrations in the adjoining areas between glomerulosa and fasciculata, and fasciculata and reticularis, with smaller elevations in the reticular-medullary zone and medulla. Better histological definition was not possible because the lower sensitivity of the chemical method necessitated the use of 8 sections per analysis. Thus reticularis, unmixed with material from adjacent zones, could not be obtained.

Although the bound pantothenate, measured microbiologically as the difference between the total and free, consists mainly of Co A, it represents a fraction somewhat different from the Co A determined by the chemical method based on the effect of Co A on enzymatic acetylation.

In the present study it is clear from Figure 1 that less than 20% of the total pantothenate in any cortical zone occurred in the free form, the con-

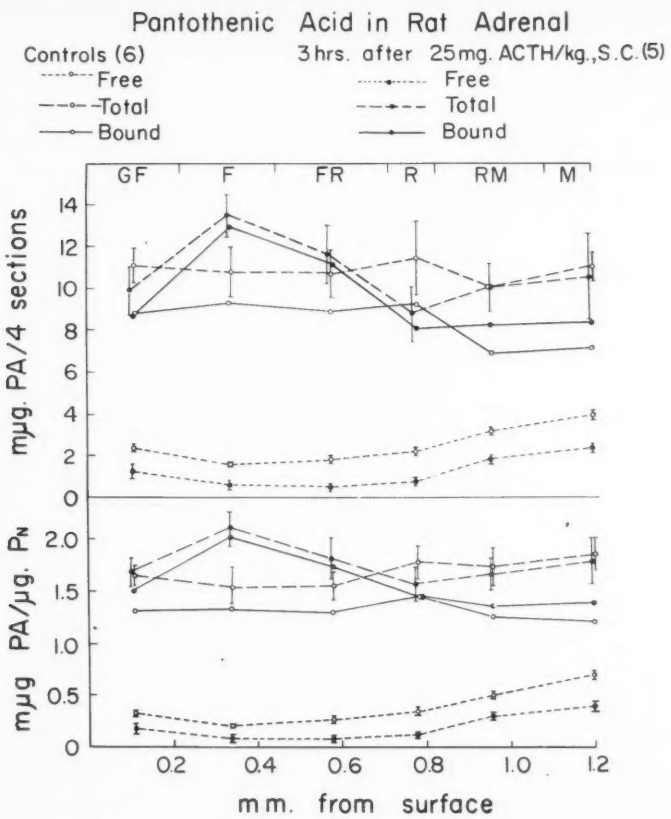

FIG. 1. Quantitative histological distribution of free, total and bound pantothenic acid (PA) in left adrenal glands of male albino rats sacrificed at noon. The numbers in parentheses denote the number of adrenals used in each case. Four microtome sections (each 16 μ thick, 2 mm. diameter) with a total volume of 0.2 μl. were used for each pantothenic acid assay (both total and free, each in triplicate, determined on the same sample). One section was used for protein-nitrogen (P_N) analysis. The points represent means, and the height of each vertical line through them represents twice the standard error of the mean. Bound pantothenate was calculated as the difference between total and free. The regions of the adrenal, identified by C, G, F, R, and M, denote, respectively, capsule, glomerulosa, fasciculata, reticularis and medulla. Mixed zones are designated by two letters.

centration of free pantothenate was rather uniform throughout the cortex but higher in medulla, and the ACTH treatment reduced the concentrations in all zones to a somewhat similar extent.

The concentration of total pantothenate in adrenals from control animals also varied little among the various zones, but after the ACTH treatment a significant rise occurred only in the fasciculata. The bound form

in control adrenals was also distributed rather uniformly in all zones, and ACTH elicited an elevation in fasciculata which was the resultant of the combined fall in free and rise in total pantothenate. Apparently a conversion of free to bound pantothenate occurred, but this accounted for only a part of the increase observed. ACTH treatment appears to effect a fascicular mobilization of Co A in addition to a direct synthesis from free pantothenate in this zone.

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MICRO-DETERMINATION OF CARBONIC ANHYDRASE ACTIVITY IN ANIMAL TISSUE[1]

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ABSTRACT

A modified manometric micro-method for the determination of carbonic anhydrase activity, using a pure carbonic anhydrase preparation for the definition of unit enzyme activity, was examined and established as a sensitive, accurate and practical method suitable for biological studies.

Estimations of the carbonic anhydrase activity in the uteri of some mammalian species indicate that this method is useful for further investigations of this enzyme.

AFTER the discovery by Lutwak-Mann (1) that gonadotrophins, progesterone and certain other luteoids produced a marked increase in the concentration of uterine carbonic anhydrase, a number of studies have been made concerning this enzyme in reproductive tracts (2–6). The colorimetric method of Philpot and Philpot (7) was used for the determination of carbonic anhydrase activity in the previous studies. However, it is a clearly established fact (8, 9, 10) that the colorimetric method, which is based on measurement of the time taken to bring about a given change in pH, is much less reliable than the original manometric method of Meldrum and Roughton (11). Moreover, the colorimetric method does not have enough sensitivity to determine the small amounts of carbonic anhydrase that are observed in certain animal tissues. In order to carry out further studies on this enzyme, the use of the Warburg apparatus is recommended because of its accuracy, sensitivity and relative simplicity (12, 13, 14).

In the present studies Warburg's method, developed by Krebs and Roughton (12), was modified and used as a simpler and more practical method suitable for the routine biological analysis of carbonic anhydrase activity.

EXPERIMENTAL PROCEDURE

General Technique

Warburg flasks of about 15 ml. capacity were used having a side arm but no center well. A magnetic stirrer was used for the stirring of the flask fluids instead of shaking by the usual Warburg apparatus. The magnetic stirrer moved two stirring bars, one in the reaction flask and the other in the ice-water bath. By this simple arrangement,

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using the magnetic stirrer, the essential conditions for the present work: efficient stirring of the reaction fluids and constant temperature at 0° C, were satisfied.

The reagents used were as follows:

(1) 0.1M-Phosphate buffer prepared by mixing 300 ml. of 0.1M-Na_2HPO_4 with 200 ml. of 0.1M-KH_2PO_4.

(2) 0.1M-$NaHCO_3$.

(3) Carbonic anhydrase solution.

2 ml. of the phosphate buffer, and additions such as carbonic anhydrase solution and distilled water in a total volume of 0.2 ml., were measured into the main compartment, and 1 ml. of the bicarbonate intc the side arm. The flasks were then placed in the ice bath and the stirrer engaged at a moderate speed. After equilibrium was established, the contents of the side arm of the flask was mixed into the fluid in the main chamber, and the rate of stirring was set at maximum. The measured change in pressure of CO_2 within the closed system was corrected for changes in the thermo-barometer. In order to keep the stirring constant, a voltage stabilizer should be used if necessary.

Carbonic Anhydrase Solution

In the preliminary experiments water-extracts of the endometria of rabbits treated with progesterone were used as the carbonic anhydrase solution. These extracts were prepared as described in a previous paper (4).

A standard carbonic anhydrase preparation[2] was used for definition of unit enzyme activity. This was a white powder purified from ox blood cells by the method of Keilin and Mann (15) and showed activity of 700,000 units/gm. by the electrometric method of Wilbur and Anderson (9).

Preparation of the Enzyme Extract from Animal Tissues

Uterine tissues from immature albino rabbits weighing approximately 1.5 kg., adult Sprague-Dawley albino rats and 50-day old albino mice, and human biopsy endometria were used for the estimation of enzyme concentration in animal tissues. All tissues were rinsed with cold saline and homogenized with adequate amounts of cold distilled water, and the homogenates were centrifuged at 1500 r.p.m. for about fifteen minutes. The supernatant was used for the determination of enzyme activity.

RESULTS AND DISCUSSION

Preliminary Study on Co_2 Evolution as Influenced by Time of Reaction and Enzyme Concentration

The first study involved the determination of CO_2 output by a mixture of phosphate buffer and bicarbonate. After establishing this point, the acceleration of CO_2 output by various amounts of carbonic anhydrase was studied. The results showed increases in CO_2 output following addition of various amounts of carbonic anhydrase, just as described by Krebs and Roughton (12) (Table 1).

Analysis of the Relationship between the Amount of Carbonic Anhydrase and the Volume of CO_2 Evolved from a Mixture of Phosphate Buffer and Bicarbonate

In order to check the relationship between the amount of carbonic anhydrase and the volume of CO_2 evolved and also the reproducibility of th

[2] K & K Laboratories, Inc. (Jamaica, New York).

TABLE 1. EXPERIMENTAL DATA ON THE EFFECT OF VARIOUS QUANTITIES OF CARBONIC ANHYDRASE ON THE RATE OF CO_2 EVOLUTION FROM A MIXTURE OF PHOSPHATE BUFFER AND BICARBONATE

Time after mixing (min.)	CO_2 evolved in μl.						
	Without enzyme	With enzyme*					
		0.05	0.10	0.15	0.20	0.30	0.60
0.5	20.6	27.0	42.9	48.1	66.3	112.2	183.3
1.0	44.8	58.6	87.2	109.5	139.6	197.9	306.3
1.5	73.2	92.5	134.9	174.4	208.3	273.4	364.4
2.0	99.3	126.4	177.9	225.8	268.8	335.2	403.6
2.5	125.9	160.2	216.1	270.5	319.2	393.6	445.9
3.0	150.7	190.8	254.3	310.8	371.9	434.8	476.2
4.0	197.9	243.8	311.6	380.1	433.7	487.5	525.4
5.0	238.6	287.7	364.1	435.9	482.9	524.0	558.9
6.0	—	327.7	405.9	475.1	—	548.0	577.9
7.0	304.8	—	—	500.8	538.4	—	592.4
8.0	—	391.1	470.4	—	—	572.1	601.4
10.0	385.1	436.0	517.0	542.6	571.0	583.3	609.2
15.0	476.7	517.0	569.5	575.7	591.6	600.7	614.9
20.0	528.5	561.8	583.5	590.8	601.3	604.1	614.9
25.0	563.5	590.2	599.6	598.4	604.4	612.5	614.9
30.0	586.4	603.8	608.5	606.2	604.4	612.5	—
35.0	599.6	611.6	608.5	607.5	604.4	612.5	—
40.0	609.4	611.6	608.5	607.5	—	—	—
45.0	614.5	611.6	—	—	—	—	—
50.0	614.5	—	—	—	—	—	—

* Enzyme extract from the endometria of progesterone-treated rabbit uteri was used. (Carbonic anhydrase extract—(A)) Expression of the enzyme amount:

0.05 = Diluted 1/10, 0.05 ml.
0.10 = Diluted 1/10, 0.10 ml.
0.15 = Diluted 1/10, 0.15 ml.
0.20 = Diluted 1/10, 0.20 ml.
0.30 = Diluted 1/5, 0.15 ml.
0.60 = Diluted 1/2.5, 0.15 ml.

results, measurements were made on four consecutive days using nine carbonic anhydrase concentrations each day (Table 2).

Krebs and Roughton (12), using a purified enzyme preparation from ox blood cells, showed that the acceleration caused by carbonic anhydrase was proportional to enzyme concentration in a narrow range of low enzyme concentrations. The time course of their reaction was 30 to 60 seconds. The same type of result was obtained in the present experiments using the crude enzyme extract of rabbit endometria; however, there was larger deviation in observed CO_2 output at 30 or 60 seconds than at the longer time course (1.5–2.0 min.). This could partially be accounted for by the technical difficulty of proper mixing and initiation of stirring.

It was found by examining the data (Table 2) that a reliable linear relationship was obtained between the logarithm of the amount of carbonic anhydrase and the volume of evolved CO_2 at a time course of 1.5 or 2.0 minutes over a wider range of enzyme concentration than that described by Krebs and Roughton (Fig. 1).

Statistical analysis gave evidence of the linearity and reproducibility of both lines obtained at a time course of 1.5 and 2.0 minutes. Taking into

TABLE 2. RELATIONSHIP BETWEEN THE AMOUNT OF CARBONIC ANHYDRASE AND THE VOLUME OF CO_2 EVOLVED FROM A MIXTURE OF PHOSPHATE BUFFER AND BICARBONATE

Time after mixing (min.)	Day of expt.	CO_2 evolved in μl.								
		Without enzyme	With enzyme*							
			0.05	0.075	0.10	0.15	0.20	0.30	0.40	0.50	0.60
0.5	1	20.9	22.1	34.7	45.8	56.2	73.7	92.9	110.7	124.8	150.3
	2	17.9	22.8	31.7	41.4	48.9	72.1	85.0	113.1	126.4	161.3
	3	20.4	24.0	34.8	41.2	54.7	74.4	95.9	123.0	133.4	156.5
	4	21.3	19.9	33.8	38.4	50.3	68.9	92.5	117.6	123.4	150.7
	Average	20.6	22.2	33.7	41.7	52.5	72.8	91.6	116.1	127.0	154.7
1.0	1	41.2	48.9	83.1	101.8	126.5	148.7	182.2	205.6	230.0	255.1
	2	40.3	54.5	74.9	91.7	123.0	156.8	169.9	213.0	222.8	277.4
	3	52.9	48.9	78.9	96.1	125.7	151.0	187.8	221.6	234.4	257.4
	4	44.7	51.5	75.6	92.8	120.3	146.5	181.5	211.6	227.6	253.2
	Average	44.8	50.9	78.1	95.6	123.9	150.7	180.3	215.4	228.7	260.8
1.5	1	75.5	82.8	125.9	157.9	191.5	219.1	269.1	286.6	312.4	342.4
	2	67.1	89.3	121.8	147.6	180.8	222.0	245.9	297.0	302.9	352.9
	3	78.9	84.4	123.0	148.8	191.0	222.0	271.9	300.6	324.9	329.5
	4	71.5	86.6	119.5	147.9	179.9	217.6	258.2	290.9	308.6	323.8
	Average	73.2	85.8	122.6	150.5	185.8	220.2	261.3	293.8	312.2	337.2
2.0	1	99.5	111.9	170.3	209.4	246.0	281.7	336.4	342.3	376.4	403.6
	2	92.8	124.1	168.9	199.0	242.2	283.7	309.6	360.4	363.2	404.8
	3	106.4	118.8	159.0	195.4	236.9	275.3	324.7	363.2	384.1	400.1
	4	98.4	120.9	163.7	199.1	251.6	283.8	337.6	354.8	375.2	386.7
	Average	99.3	118.9	165.5	200.7	244.2	281.1	327.1	355.2	374.7	398.8
2.5	1	127.0	139.9	203.0	252.8	291.3	334.3	379.5	383.8	425.7	446.1
	2	119.6	155.0	205.7	243.7	287.1	336.4	363.4	408.9	411.9	447.7
	3	131.6	152.1	190.3	241.4	302.8	332.9	384.6	415.4	437.4	423.4
	4	125.2	151.0	196.2	234.7	285.0	328.6	376.6	398.9	422.9	436.7
	Average	125.9	149.5	198.8	243.2	291.5	333.1	376.0	401.7	424.5	438.5
3.0	1	152.2	167.9	235.7	295.2	333.5	370.0	417.7	434.8	460.0	446.1
	2	145.3	185.9	243.7	286.2	322.8	375.3	401.3	440.1	454.9	481.5
	3	155.6	183.2	221.6	282.6	341.5	375.3	427.3	456.0	474.6	461.1
	4	149.8	176.5	233.4	278.2	329.8	374.1	417.2	453.5	460.7	467.1
	Average	150.7	178.4	233.6	285.5	331.9	373.7	415.9	446.1	462.3	471.5
4.0	1	196.9	216.8	289.8	352.4	395.0	444.9	476.9	495.8	518.4	525.4
	2	196.8	240.7	309.6	351.0	389.2	446.3	474.0	499.5	509.4	527.7
	3	202.5	237.5	288.9	346.7	408.8	439.5	483.2	509.4	521.0	514.9
	4	195.7	229.8	289.8	338.3	401.3	437.4	477.0	501.2	507.4	514.8
	Average	197.9	231.2	294.5	347.1	398.6	442.0	477.8	501.5	514.1	520.7

* Carbonic anhydrase extract—(A) was used as noted in Table 1.

account the regression coefficients of both lines and the elimination of th technical difficulty above mentioned, a 2.0 minute time course was adopte in the following experiments. Statistical analysis of the 2.0 minute tim course is shown in Table 3.

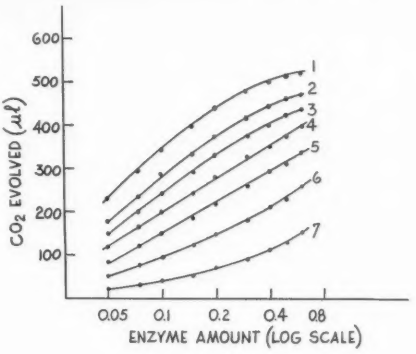

FIG. 1. Relationship between the amount of carbonic anhydrase and the volume of CO_2 evolved from a mixture of phosphate buffer and bicarbonate.

Carbonic anhydrase extract—(A) was used as noted in Table I.

Time course of reaction:

1 = 4.0 min.
2 = 3.0 min.
3 = 2.5 min.
4 = 2.0 min. (Y = 258.4 log.X − 136.8)
5 = 1.5 min. (Y = 232.9 log.X − 151.6)
6 = 1.0 min.
7 = 0.5 min.

Standard Curve

Pure carbonic anhydrase preparation was used for the definition of an enzyme unit.

From the data of two sets of determinations as shown in Table 4, the standard curve of the relation between volume of CO_2 evolved and enzyme units was obtained (Fig. 2). When working with crude extracts of animal tissue, the inactivation of carbonic anhydrase must be considered (16). However, the slope of the standard curve (b = 259.2) of the purified preparation coincided perfectly with that of the curve obtained from the enzyme extract of the rabbit endometrium (b = 258.4). This fact points to the reliability and usefulness of this method for biological experimentation.

Indirect Determination of Enzyme Activity in lower Concentration

Enzyme activity between 0.25 unit/0.2 ml. and 1.5 unit/0.2 ml. was measurable by the above described standard curve. However, the indirect determination of enzyme activity at even lower concentration was made possible by using the standard carbonic anhydrase preparation for the definition of unit enzyme activity. An enzyme solution having less ac-

TABLE 3. STATISTICAL ANALYSIS OF LINEARITY AND REPRODUCIBILITY
(Reaction time: 2.0 minutes)

Factor	Sum of sq.	d.f.	Variance
Linearity	308,758.21	1	
Residue	292.62	7	41.80
Dose	309,050.	8	
Day	359.12	3	119.71
Error	1,271.34	24	52.97
Total	310,681.29	35	

$F_1 = 0.7891$; $F_{24}^7 = 5.5$ at $P = 0.001$,
$F_1 \ll F_{24}^7$ shows conclusive linearity. (Y = 258.4 log.X − 136.8)
$F_2 = 2.278$; $F_{24}^3 = 7.55$ at $P = 0.001$,
$F_2 \ll F_{24}^3$ shows excellent reproducibility.

TABLE 4. DETERMINATION OF THE STANDARD CURVE
(Aqueous solution of pure carbonic anhydrase preparation)

Enzyme unit	CO_2 evolved in μl. (Reaction time: 2.0 min.)
0.25	104.8, 115.7
0.375	155.1, 159.6
0.5	187.3, 181.7
0.75	243.1, 229.2
1.0	268.1, 262.5
1.5	316.8, 310.0

Formula of the line: $Y = 259.2 \log X + 6.8$.

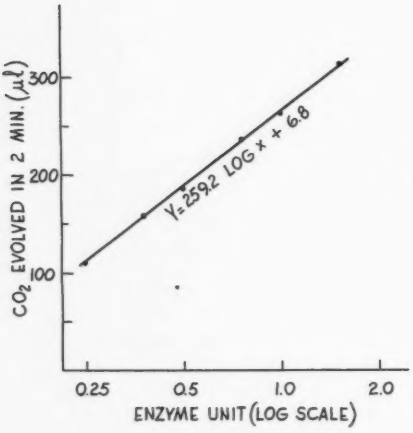

FIG. 2. Standard curve.

TABLE 5. INDIRECT DETERMINATION OF ENZYME ACTIVITY

Enzyme solution	Volume of enzyme sol. used (ml.)	CO_2 evolved (μl.)	E.U./0.2 ml.
1. Standard Carbonic Anhydrase Solution (S), Diluted 1/25	0.2	174.3	0.445*
2. Extract No. 121** (a) Original Solution	0.02	166.0	4.12
(b) Diluted 1/30	0.2	105.3	—
(c) 4.8 ml. (b) +0.2 ml. (S)	0.2	210.4	0.61
Calculated value of (b) from (c) and (S)			*0.158*
Calculated value of (b) from (a)			*0.137*
3. Extract No. 123** (d) Original Solution	0.02	228.1	7.10
(e) Diluted 1/30	0.2	116.0	0.26
(f) 4.8 ml. (e) +0.2 ml. (S)	0.2	227.1	0.707
Calculated value of (e) from (f) and (S)			*0.252*
Calculated value of (e) from (d)			*0.237*

* Theoretical value = 0.45 E.U./0.2 ml.
** Extract of rabbit endometrium.

ivity than 0.25 unit/0.2 ml. can be measured after the addition of a known nd adequate amount of the standard carbonic anhydrase preparation. The xperimental data in Table 5 show the results of such determinations. In his way it is possible to estimate the enzyme activity in concentrations as ow as 0.05 unit/0.2 ml.

Carbonic Anhydrase Activity in the Uteri of Some Mammalian Species

The present micro-determination of carbonic anhydrase activity was applied to the investigation of the enzyme content of the uteri of normal adult mice, adult rats, immature rabbits and endometria of humans and rabbits.

TABLE 6. CARBONIC ANHYDRASE CONTENT OF SOME MAMMALIAN UTERI

Species	Tissue	No. of samples	Uterine weight*	E.U./g. tissue*
Mouse	Whole Uteri	35	51.2 ± 6.7	364.0 ± 17.0
Rat	Whole Uteri	24	387.3 ± 26.9	35.0 ± 1.8
Rabbit	Whole Uteri	4	372.2 ± 41.1	61.7 ± 5.8
Rabbit	Whole Uteri (E)**	4	1878.2 ± 229.0	26.0 ± 2.9
Rabbit	Endometrium (E)**	10	38.8 ± 5.3	157.8 ± 17.7
Human	Endometrium	5	(16.0 – 89.0)***	127.8 ± 13.5

* Mean ± Standard Error.
** Rabbits were treated with 5 μg. Estradiol (E) daily for 5 days.
*** Biopsy specimens.

The results are summarized in Table 6. The uterus of the mouse contains exceptionally high concentrations of carbonic anhydrase compared with those of rats and rabbits. It was also observed that the carbonic anhydrase content of human biopsy endometria is quite high. The enzyme content of the mouse uterus and the human endometrium has not been reported in previous studies. The data of Table 6 demonstrate also (a) a higher enzyme concentration in the uterine endometrium of the rabbit than in the whole uterus, but (b) a lowered concentration in the estrogen-treated than in the untreated immature rabbit uterus (17), and (c) the possibility of adequately accurate enzyme determinations in human endometrial biopsy specimens.

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THE ENDOCRINE SOCIETY

The 1961 Annual Meeting

The Forty-Third Annual Meeting of the Endocrine Society will be held in the Hotel Biltmore, New York, N. Y., Thursday, Friday and Saturday, *June 22, 23 and 24, 1961.*

The Chairman of Local Arrangements is Dr. Joseph W. Jailer.

The Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 23, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Biltmore will hold 300 bedrooms for members until May 1, 1961, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 26 for the American Medical Association and other meetings, it is imperative that your reservations be made from June 26 on directly through the A.M.A. Housing Bureau. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1961.

Final program, membership card and advance registration forms will be sent on May 1, 1961 to members whose current dues have been paid.

1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members

of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959 and Dr. Emil Witschi in 1960. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. James A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell and 1960 Dr. Don H. Nelson. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.

4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15 each year.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the secretary of the Society. They should be submitted by October 15 each year.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.

AMERICAN PSYCHOSOMATIC SOCIETY

The American Psychosomatic Society will hold its 18th Annual Meeting at Chalfonte-Haddon Hall in Atlantic City on Friday, Saturday, and Sunday, April 28, 29, and 30, 1961.

The Program Committee would like to receive titles and abstracts of papers for consideration for the program. Time allotted for each presentation of each paper will be twenty minutes. The deadline for the submission of abstracts is December 1, 1960; and the abstracts should be not more than two typewritten pages in length. The Program Committee would like to receive nine copies of each abstract.

Abstracts submitted for the Program Committee's consideration should be addressed to the Chairman, at 265 Nassau Road, Roosevelt, New York.

Morton F. Reiser, M.D. Chairman, Program Committee

THIRD WORLD CONGRESS OF PSYCHIATRY

The Third World Congress of Psychiatry, June 4–10, 1961, Montreal, Canada, is being held at the invitation of McGill University and under the auspices of the Canadian Psychiatric Association. Meeting on the American Continent for the first time, the Congress is expected to attract some 3,000 delegates from 62 nations. Representatives will come from psychiatry and such allied fields as general medical practice, psychology, biochemistry, nursing, sociology, anthropology, social work, and pharmacology.

Copies of the Second Announcement, which carry information regarding programme and registration, may be obtained by writing the General Secretary, III World Congress of Psychiatry, 1025 Pine Avenue West, Montreal 2, P.Q., Canada.